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Regulation of Innate Immune Cells

by

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ABSTRACT

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Immune cells such as neutrophils and monocytes enter tissues after tissue damage and clear cell debris to allow repair cells such as fibroblasts to close the wound. Monocytes also differentiate into fibroblast-like cells called fibrocytes to mediate wound healing, similar to fibroblasts. However, in abnormal wound healing such as acute respiratory distress syndrome (ARDS) and fibrosing diseases, the accumulation of immune cells such as neutrophils or fibrocytes become detrimental to health. In ARDS, neutrophils accumulate in the lungs and causes additional damage by producing reactive oxygen species (ROS). In fibrosing diseases, increased fibrocyte differentiation is one of the causes that increase extracellular matrix deposition, which leads to severe scar tissue build up. Since there are no effective treatments for ARDS or fibrosing diseases, understanding the regulation of neutrophil activation or fibrocyte differentiation could be helpful to develop new effective therapies.

The Gomer lab has found several factors that either promote or inhibit fibrocyte differentiation. The pro-fibrotic cytokines such as IL-4 and IL-13 potentiate fibrocyte differentiation while the plasma protein serum amyloid P

(SAP), crosslinked IgG, and the pro-inflammatory cytokines IFN-γ and IL-12 inhibit fibrocyte differentiation. In this thesis, I have now shown that additional factors such as toll-like receptor 2 (TLR2) agonists and low molecular weight hyaluronic acid (LMWHA) inhibit fibrocyte differentiation, while high molecular weight hyaluronic acid (HMWHA) potentiate fibrocyte differentiation.

The accumulation of neutrophils in the lungs is one of the major factors that debilitate the health of a patient in ARDS. Since neutrophils have Fc receptors, I examined the effect of SAP on neutrophil spreading, adherence, activation, and accumulation. SAP inhibits neutrophil spreading induced by cell debris and TNF-α induced neutrophil adhesion, but SAP is unable to have any effect on classic neutrophil adhesion molecules or the production of hydrogen peroxide. SAP inhibits neutrophil accumulation in the lungs of bleomycin-injured mice. There is an exciting possibility of using SAP as a therapeutic agent to treat ARDS.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

ACK	potassium bicarbonate
ALI	acute lung injury
ANOVA	analysis of variance
AP-1	activator protein 1
ARDS	acute respiratory distress syndrome
$\alpha 2\beta 1/\alpha 3\beta 1$/etc	alpha2beta1/alpha3beta1
BAL	bronchoalveolar lavage
bio-HABP	biotinylated hyaluronic acid binding protein
BSA	bovine serum albumin
CCR2	chemokine (C-C motif) receptor 2
CD	cluster differentiation
CRP	C-reactive protein
CXCR4	C-X-C chemokine receptor type 4
CysLT1	cysteinyl leukotriene receptor 1
Da	dalton
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECM	extracellular matrix
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Fab	fragment antigen-binding
Fcγ	Fc gamma receptor
FITC	fluorescein isothiocyanate
fMLP	N-formylmethionyl-leucyl-phenylalanine
G-CSF	granulocyte colony stimulating factor
GIMIQ	Gardiquimod

GM-CSF	granulocyte macrophage colony stimulating factor
HA	hyaluronic acid
HEK 293	human embryonic kidney 293
HKLM	Heat Killed *Listeria monocytogenes*
HLA-DR/DP/DQ	human leukocyte antigen-DR/DP/DQ
HMWHA	high molecular weight hyaluronic acid
hSAP	human SAP
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
IMIQ	Imiquimod
kDa	kilodalton
KRPG	Krebs-Ringer phosphate glucose buffer
LM-MS	lipomannan from *Mycobacterium smegmatis*
LMWHA	low molecular weight hyaluronic acid
LPS	lipopolysaccharide
LTA	lipotechoic acid
LYVE	lymphatic vessel endothelial hyaluronic acid receptor
Ly6G	lymphocyte antigen 6G
MAPK	mitogen-activated protein kinase
MIP	macrophage inflammatory proteins
MyD88	myeloid differentiation primary response gene (88)
mSAP	mouse SAP
MHC	major histocompatibility
μg	microgram
μl	microliter
μM	micromolar
μm	micrometer
mg	milligram
ml	milliliter

mM	millimolar
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NFκB	nuclear factor kappa B
NK	natural killer cells
NLR	nucleotide oligomerization domain-like receptor
NOD	nucleotide oligomerization domain
ng	nanogram
nM	nanomolar
OCT	optimal cutting temperature
ODN	CpG oligonucleotide
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PDBu	phorbol 12,13-dibutyrate
PFA	paraformaldehyde
pg	picogram
PG-LPS	lipopolysaccharide from *Porphyromonas gingivalis*
PGN	peptidoglycan
PMA	phorbol 12-myristate 13-acetate
Poly(I:C)	polyinosinic-polycytidylic acid
PRR	pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand-1
PTX3	pentraxin 3
PVDF	polyvinylidene difluoride
RBC	red blood cell
RHAMM	receptor for hyaluronan-mediated motility
SAP	serum amyloid P
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	standard error of mean

SFM	serum free media
ssDNA	single-stranded deoxyribonucleic acid
ssRNA	single-stranded ribonucleic acid
TBS	tris buffered saline
TGF	transforming growth factor
Th	T helper
TLR	toll-like receptor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRAM	TIR-domain-containing adapter-inducing interferon-β-related adaptor molecule
TRAF	tumor necrosis factor receptor associated factor
TRIF	TIR-domain-containing adaptor inducing interferon-β
Tris	tris(hydroxymethyl)aminomethane

Chapter 1: Fibrocytes in wound healing and fibrosing diseases, and neutrophil activation in acute respiratory distress syndrome (ARDS)

1.1 Overview and Significance

Fibrosing diseases and acute respiratory distress syndrome (ARDS) are major medical problems in many western countries (King et al., 2011; Perez et al., 2003; Wheeler and Bernard, 2007). A patient comes into the hospital with an abrasion on his legs, and within 12 hours, he has a hard time breathing and has to be put on a ventilator. He dies within 24 hours after being admitted since he is unable to breathe and has 90% influx of neutrophils in his lungs compared to a normal healthy human lungs (Bastarache and Blackwell, 2009). This is one example of the 400,000 cases of acute lung injury (ALI) or its severe form called acute respiratory distress syndrome (ARDS) that affects many people in the United States. ALI/ARDS is a condition where a patient has severe breathing problem and a huge influx into the lungs of a type of white blood cell called neutrophils. About 74,500 patients die each year from this disease, and the survivors develop long-term pulmonary fibrosis. There is only a 30% survival rate after being diagnosed with pulmonary fibrosis (King et al., 2011; Perez et al., 2003). Fibrosing diseases are characterized by scar tissue build-up, which result from excessive production of extracellular matrix proteins such as collagen and fibronectin (Wynn, 2007). Circulating monocyte-derived cells called fibrocytes have been implicated in the formation of fibrotic lesions and can potentially

differentiate into collagen producing cells or activate fibroblasts to produce more collagen (Reilkoff et al., 2011). Little is known about the progression of ALI/ARDS or fibrosing diseases. Therefore, understanding how wound healing strays away from normal healing to aberrant healing is a fundamental question in reparative medicine. In this research, I try to understand the biology of wound healing in terms of monocyte to fibrocyte differentiation and the role of neutrophils in acute inflammation.

The process of wound healing begins immediately after tissue injury. There is a systematic process in which different immune cells such as neutrophils and monocytes clear the wound to aid repair cells such as fibroblasts to repair the injured sites (Piantadosi and Schwartz, 2004). Monocyte-derived cells called fibrocytes participate in wound healing (Abe et al., 2001; Bucala, 1994; Gomperts and Strieter, 2007). In an unchecked wound healing process, there is an excessive accumulation of extracellular matrix proteins and one of the culprits for this deposition is fibrocytes (Bellini and Mattoli, 2007a; Mattoli et al., 2009; Strieter et al., 2009b). In our laboratory, we try to understand the biology of monocyte to fibrocyte differentiation and find potential therapies that can either inhibit or potentiate fibrocyte differentiation. Potentiation of fibrocyte differentiation is beneficial for people, such as diabetic patients, who have difficulty healing wounds (Ferreira et al., 2006). On the other hand, inhibition of fibrocyte differentiation is beneficial for patients who have excess scar tissue

build up because of fibrocytes (Barth et al., 2002; Bellini and Mattoli, 2007a; Haudek et al., 2006; Quan et al., 2004).

In this thesis, I elucidate the biology of fibrocyte differentiation in chapters 2 and 3. In chapter 2, I examined the different pathogen-associated molecular patterns and their roles in fibrocyte differentiation. I show that when human peripheral blood mononuclear cells (PBMC) are cultured with toll-like receptor 2 (TLR2) agonists, monocyte to fibrocyte differentiation is inhibited. The TLR2 agonists appear to stimulate some cells in the PBMC population to secrete a signal that inhibits the ability of purified monocytes to differentiate into fibrocytes. This signal is not IFN-γ, IFN-α, aggregated IgG, or serum amyloid P, signals known to directly inhibit the differentiation of monocytes to fibrocytes. These results identify a novel mechanism used to regulate the differentiation of monocytes to fibrocytes. I also examined endogenous danger signals that can affect monocyte to fibrocyte differentiation. In chapter 3, I have examined an extracellular matrix component called hyaluronic acid and how it affects monocyte to fibrocyte differentiation. In healthy tissues, hyaluronic acid is present as high molecular weight polymers, while in fresh wounds and fibrotic lesions, hyaluronic acid breaks down to low molecular weight polymers. In this research, I show that high molecular weight hyaluronic acid potentiates fibrocyte differentiation while low molecular weight hyaluronic acid inhibits fibrocyte differentiation.

In the early phase of wound healing, termed as acute inflammation, immune cells such as neutrophils and monocytes enter the injured sites. In a normal wound healing situation, these immune cells are cleared out of the wound site after accomplishing their tasks of engulfing bacteria or clearing out cell debris. However, in acute lung injury (ALI) or acute respiratory distress syndrome (ARDS), immune cells such as neutrophils accumulate in the injured site and cause additional damage to the wound. In our laboratory we found that the plasma protein serum amyloid P (SAP) can inhibit fibrocyte differentiation through Fc receptors. Since neutrophils also have Fc receptors, I examined the effect of SAP on neutrophils *in vitro* and *in vivo*. Neutrophil activation leads to increased adherence, increased surface levels of the adhesion molecules, increased production of hydrogen peroxide, and increased accumulation of neutrophils in bleomycin treated mice lungs (Montecucco et al., 2008; Nathan et al., 1989; Nathan, 1987, 1989; Weisbart et al., 1985; Weisbart et al., 1982; Yong et al., 1992). In chapter 4, I show that SAP diminishes the adhesion of neutrophils, but has no effect on classic neutrophil adhesion molecules or the production of hydrogen peroxide. I also show that SAP inhibits the accumulation of neutrophils in bleomycin treated mice lungs.

1.2 Tissue Injury and Wound Healing

Following tissue injury, local repair cells called fibroblasts proliferate to repair the wound (Martin, 1997; Singer and Clark, 1999). In addition to fibroblasts, circulating peripheral blood cells such as neutrophils, monocytes, dendritic cells and lymphocytes leave the bloodstream and enter the injured site. Once monocytes are in the injured site, they can differentiate into fibroblast-like cells called fibrocytes that also aid in healing (Abe et al., 2001; Bucala, 1994; Gomperts and Strieter, 2007; Haudek et al., 2006; Pilling et al., 2003; Pilling et al., 2006; Quan et al., 2004; Yang et al., 2002). Fibrocytes can differentiate from purified $CD14^+$ peripheral blood monocytes, but fibrocytes lose expression of CD14 (Abe et al., 2001; Maharjan et al., 2010; Pilling et al., 2003; Shao et al., 2008; Yang et al., 2002). Other studies also suggest that fibrocytes differentiate from a population of bone-marrow derived $CD45^+$ $CXCR4^+$ cells found in peripheral blood (Phillips et al., 2004; Reilkoff et al., 2011; Strieter et al., 2009a). Fibrocytes are spindle-shaped cells that express hematopoietic cell markers such as MHC class II, CD34, CD45RO, 25F9, and S100A8/A9, stromal cell markers such as collagen I, and collagen III, and chemokine receptors such as CCR2, CXCR4, and CCR7 that mediate their entry into the site of injury (Bucala, 1994; Chesney, 1997; Hashimoto et al., 2004; Moore et al., 2005; Phillips et al., 2004; Pilling et al., 2009). Fibrocytes produce cytokines, collagens, angiogenic and fibrogenic growth factors, and matrix metalloproteinases that help to rebuild tissue after injury (Abe et al., 2001; Bucala, 1994; Gomperts and Strieter, 2007;

Hartlapp et al., 2001; Mori et al., 2005; Quan et al., 2004; Wang et al., 2007b; Yang et al., 2002). Fibrocytes are found as a circulating population of cells present in the peripheral blood, and there are elevated numbers of fibrocytes in patients with inflammatory and fibrotic diseases (Bellini and Mattoli, 2007b; Mattoli et al., 2009; Strieter et al., 2009a).

1.3 Fibrosing diseases

The inability to form scar tissues in chronic wounds, and the unwanted appearance of scar tissue in fibrosing diseases, are major medical problems. In many western countries, fibrosing diseases like congestive heart failure, kidney failure, cirrhosis of the liver, idiopathic pulmonary fibrosis, and scleroderma cause chronic and fatal disorders in many people (King et al., 2011; Perez et al., 2003; Wynn, 2007). Fibrosis is described as an out of control wound repair that leads to an excessive growth of repair tissue. In cases of repeated injuries, inflammation and repair processes alters the cytokine level, which causes a failure to degrade extracellular matrix (ECM) proteins, and therefore, leads to increased deposition of ECM and thus fibrosis (Cohn et al., 2004; Powell et al., 1999; Wynn, 2004).

1.4 Role of fibrocytes in fibrosing diseases

Recent studies show the role of fibrocytes not only in wound repair, but also in diseases like breast cancer, scleroderma, and pulmonary fibrosis and

congestive heart failure (Barth et al., 2002; Haudek et al., 2006; Quan et al., 2004; Schmidt et al., 2003). Therefore, inhibiting fibrocytes could be a way to reduce the excessive ECM formation in most of these diseases.

1.5 Factors that regulate fibrocyte differentiation

We found that the plasma protein serum amyloid P (SAP) can directly inhibit monocytes from differentiating into fibrocytes (Pilling et al., 2003). Purified SAP inhibits fibrocyte differentiation, but other serum proteins such as serum amyloid A and C-reactive protein (CRP) are unable to inhibit the differentiation of monocytes into fibrocytes (Pilling et al., 2003; Pilling, 2007). The profibrotic cytokines interleukin (IL)-4 and IL-13 directly activate monocytes to differentiate into fibrocytes, while cross-linked immunoglobulin G (IgG) and the proinflammatory cytokine interferon (IFN)-γ directly inhibit the differentiation of monocytes into fibrocytes (Haudek et al., 2006; Shao et al., 2008). Another proinflammatory cytokine, IL-12, activates some cells in the peripheral blood mononuclear cell (PBMC) population, possibly natural killer (NK) cells, to indirectly inhibit fibrocyte differentiation (Shao et al., 2008). In human PBMC culture, IFN-α2b inhibits fibrocyte differentiation, but whether this acts directly on monocytes is unknown (Chang et al., 2007). Other regulators of fibrocytes include the adenosine A_{2A} receptor and cysteinyl leukotriene receptor 1 (CysLT1) (Katebi et al., 2008; Vannella et al., 2007). The adenosine A_{2A} receptor regulates cell proliferation and cytokine production, and blocking this receptor inhibits the

recruitment of fibrocytes in bleomycin-treated mouse skin (Katebi et al., 2008). CysLT1 is a receptor for lipid mediators which promote fibroblast proliferation, fibroblast chemotaxis and collagen synthesis (Vannella et al., 2007). In mice with fluorescein isothiocyanate (FITC)-induced lung fibrosis, blocking CysLT1 inhibits the appearance of fibrocytes (Vannella et al., 2007).

It is unclear why some of the above factors affect fibrocyte differentiation. However, we hypothesized that SAP prevents fibrocyte differentiation in the circulation and that aggregated IgGs prevent fibrocyte differentiation because aggregated IgGs signify the presence of infection. We hypothesized that after monocytes detect an infected wound, monocytes do not differentiate into fibrocytes, since closing an infected wound could cause further damage such as gangrene, an infectious wound closure that results in further decay of the surrounding cells (Ferreira et al., 2006; Safioleas et al., 2006).

1.6 Acute respiratory distress syndrome (ARDS)/acute lung injury (ALI)

Smoke inhalation, for instance from house fires, can lead to severe lung damage called acute lung injury (ALI) or acute respiratory distress syndrome (ARDS). ARDS can be caused by infection, sepsis, aspiration (for example stomach contents), and trauma to the lungs. In a typical case, a patient develops severe breathing problems 48 to 96 hours after the smoke inhalation. In the United States, there are 400,000 cases of ALI/ARDS per year (Baudouin, 2004;

Chabot et al., 1998; Piantadosi and Schwartz, 2004). Due to the lack of effective treatments, the mortality rate remains as high as 40% (Wheeler and Bernard, 2007).

In many ALI/ARDS patients, huge amounts of debris from damaged lung cells causes a massive accumulation of activated neutrophils into the lungs (Chollet-Martin et al., 1996; Chopra et al., 2009; Weiland et al., 1986). Unfortunately, this massive influx of activated neutrophils is one of the major factors that contribute to the progression of the disease (Segel et al., 2011). Activated neutrophils continue to release reactive oxygen species and proteases that damage the remaining lung cells, causing a vicious cycle of damage, neutrophil influx and activation, and further neutrophil-induced damage (Perl et al., 2008; Segel et al., 2011).

Chapter 2: Toll-like receptor 2 agonists inhibit human fibrocyte differentiation

2.1 Introduction

The immune system can recognize pathogens using Toll-like receptors (TLRs) (Akira et al., 2003; Beutler, 2009; Hawn et al., 2007; Janeway and Medzhitov, 2002; Medzhitov and Janeway, 2000; Medzhitov and Janeway, 1999; Trinchieri and Sher, 2007). There are 11 different TLRs in humans and 16 different TLRs in mouse (Figure 2.1). TLR agonists include pathogen-specific molecules such as lipopolysaccharides (LPS) from gram-negative bacteria (recognized by TLR4), flagellin from bacteria (recognized by TLR5), single-stranded DNA (ssDNA) from viruses (recognized by TLR8), and unmethylated DNA from bacteria (recognized by TLR9) (Janeway and Medzhitov, 2002; Medzhitov and Janeway, 1998). TLR2 recognizes a wide variety of molecular patterns such as peptidoglycan, lipotechoic acid, and lipoproteins from gram-positive bacteria, zymosan from the yeast cell wall, and lipoarabinomannan from mycobacteria (Hawn et al., 2007; Lien et al., 1999; Medzhitov and Janeway, 2000; Yoshimura et al., 1999). TLRs 1, 2, 4, 5, and 6 are specialized to recognize bacterial products and are situated on the plasma membrane of a cell (Figure 2.1.1). Most of the TLRs homodimerize and recognize the microbial molecular patterns, except for TLR2. TLR2 can heterodimerize with either TLR1 or TLR6 and recognize different bacterial lipoproteins (Lien et al., 1999;

Yoshimura et al., 1999). TLR2 heterodimerizes with TLR1 and detects triacylated lipoprotein, whereas TLR2 detects diacylated lipoprotein as it forms a heterodimer with TLR6. This gives TLR2 a unique ability to detect various bacterial patterns. On the other hand, TLRs 3, 7, 8, and 9 are specialized to detect viral compounds such as viral DNAs and ssRNAs, and these receptors are localized in intracellular compartments (Figure 2.1.1). TLR signaling pathways trigger innate immune responses through nuclear factor (NF)-κB-dependent and IFN-regulatory factor-dependent pathways (Trinchieri and Sher, 2007).

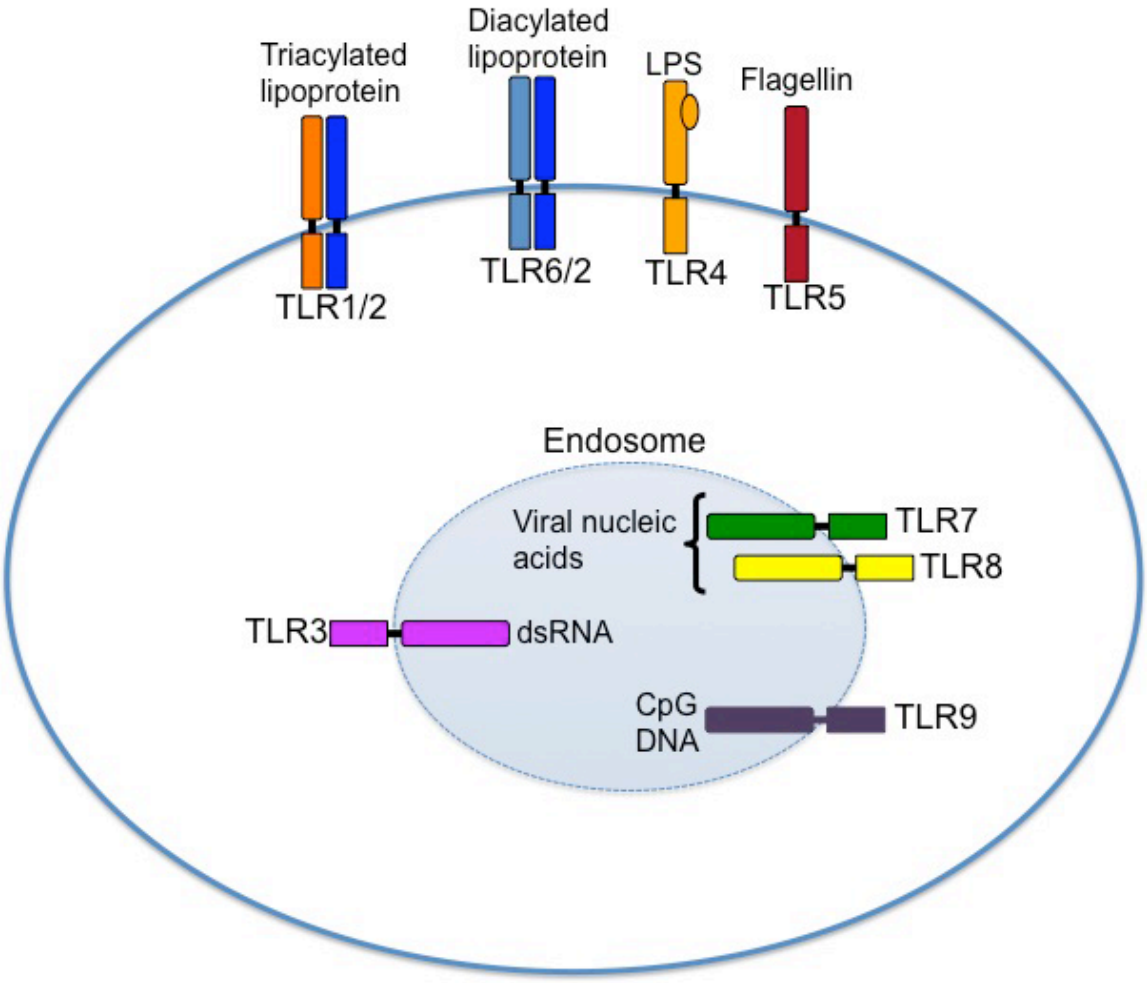


Figure 2.1.1 The toll-like receptors (TLRs) and their pathogen specific recognition patterns (modified from (Takeda and Akira, 2004)). TLRs 1, 2, 4, 5, and 6 are located on the plasma membrane of the cell and these TLRs are specialized to recognize bacterial peptides and lipopolysaccharides. TLRs 3, 7, 8, and 9, on the other hand, are located in the endosomal compartments and these TLRs are specialized to recognize various viral nucleic acids.

TLRs are present in many immune cells such as NK cells, dendritic cells, monocytes, B cells, and T cells (Table 2.1.1) (Hornung et al., 2002; Zarember and Godowski, 2002).

Table 2.1.1 mRNA expression of TLR1-10 in different immune cells* (Hornung et al., 2002; Zarember and Godowski, 2002)**.**

	Monocytes	Plasmacytoid Dendritic Cells	NK cells	B cells	T cells
TLR1	Moderate	Low	High	High	Moderate
TLR2	High	None	Moderate	Low	Low
TLR3	None	None	Moderate	None	Low
TLR4	High	None	Low	Low	None
TLR5	Moderate	None	Moderate	None	Moderate
TLR6	Low	Low	Moderate	High	Low
TLR7	Low	Moderate	Low	Low	Low
TLR8	Moderate	None	Low	None	Low
TLR9	Low	High	Low	Moderate	Low
TLR10	None	Low	Low	Moderate	None

*The intensity of the mRNA expression levels of TLRs in monocytes, plamacytoid dendritic cells, NK cells, B cells, and T cells is shown.

From our previous studies, we hypothesized that aggregated IgG and SAP signal monocytes of the presence of sepsis, and therefore, prevents wound closure by inhibiting fibrocyte differentiation. Since TLRs are present on monocytes (Table 2.1.1) and they too recognize the presence of infection, we examined whether TLR agonists could also affect the differentiation of monocytes into fibrocytes.

2.2 Methods

2.2.1 Culturing PBMC with TLR agonists or IFN-α

Blood was collected from healthy adult volunteers in accordance with specific approval of Rice University's Institutional Review Board. Written consent was received from all volunteers, and all samples were deidentified before analysis. PBMCs were isolated and incubated in serum-free media (SFM) as described previously (Pilling et al., 2003; Pilling et al., 2006; Pilling et al., 2009; Shao et al., 2008). TLR agonists (Invivogen, San Diego, CA, USA) were reconstituted in endotoxin-free water (Invivogen). All experiments were done with at least three different batches of agonists. IFN-α was obtained from EMD-Calbiochem (Darmstadt, Germany). A dilution series of TLR agonists (or the same volume of water as a control) was made in serum-free medium. A quantity of 100 μl of serum-free medium, TLR agonist or IFN-α dilution, or water dilution, was added to duplicate wells of a 96-well tissue culture plate (BD Biosciences, San Jose, CA, USA). A quantity of 100 μl of human PBMCs at a concentration of 5×10^5 cells/ml in serum-free medium was then added to each well. On day 5, fields of PBMCs were photographed using a phase-contrast microscope, and the number of cells per image was counted. Cells were then fixed and stained, and fibrocytes were counted as previously described (Pilling et al., 2003; Pilling et al., 2006; Pilling et al., 2009; Shao et al., 2008).

2.2.2 Preparation of monocytes

Monocytes were purified from 5×10^7 PBMCs using an EasySep Monocyte Depletion Kit (catalog no. 19059; StemCell Technology, Vancouver, BC, Canada) according to the manufacturer's instructions. To determine the purity of the monocytes, cells were analyzed using flow cytometry (FACScan, BD Biosciences; or Accuri C6 cytometer, Accuri Cytometers Inc., Ann Arbor, MI, USA) as described previously (Shao et al., 2008). A sample of each monocyte preparation was stained with 5 µg/ml primary antibodies against the T cell marker CD3, the monocyte marker CD14, the NK cell marker CD16, the B cell marker CD19 and the leukocyte marker CD45 as previously described (Shao et al., 2008). Monocytes obtained were greater than 95% pure as determined by the expression of CD14. Monocytes were 99% CD45-positive, 0.93% CD3-positive, 0.93% CD16-positive and 1% CD19-positive. To assess the effect of TLR2 agonists on monocytes, 5 µl of TLR2 agonist or water were added to 245 µl of serum-free medium. A quantity of 100 µl of serum-free medium, TLR2 agonist dilution or water dilution was added to a well of a 96-well plate, with each condition represented in duplicate wells. A quantity of 100 µl of purified human monocytes at a concentration of 5×10^5 cells/ml in serum-free medium was then added to each well. Fibrocytes were fixed, stained and counted after monocytes were cultured for 5 days.

2.2.3 Treating monocytes with conditioned media from PBMC stimulated with TLR2 agonists

To make conditioned medium, a dilution of TLR2 agonists was made as described above. A quantity of 100 µl of either SFM, the TLR2 agonist dilution or the corresponding water dilution was added to duplicate wells of a 96-well plate along with 100 µl of PBMCs at a concentration of 5×10^5 cells/ml in SFM. After 3 days of incubation, 180 µl of the conditioned media from one well representing each condition were transferred into an Eppendorf tube, snap-frozen in liquid nitrogen and stored at -20°C. For the well from which the medium was not removed, at day 5 the fibrocytes were fixed, stained and counted as previously described (Pilling et al., 2003; Pilling et al., 2006; Shao et al., 2008). Also on day 5, monocytes were prepared from the same donor as described above. A quantity of 50 µl of monocytes at a concentration of 1×10^6 cells/ml was incubated with 50 µl of the day 3 conditioned medium. After 5 days, cells were fixed and stained, and the number of fibrocytes was counted.

2.2.4 Detection of cytokines and SAP by enzyme-linked immunosorbent assay

The day 3 conditioned media were analyzed for IFN-α, IFN-γ and IL-12 using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Peprotech, Rocky Hill, NJ, USA). The day 3 conditioned media were also analyzed for IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17A, IFN-γ, TNF-α, granulocyte colony-stimulating factor (G-CSF) and transforming growth factor

(TGF)-β1 using a multi-analyte profiler ELISArray kit according to the manufacturer's instructions (SABiosciences, Frederick, MD, USA).

The day 3 conditioned media were also analyzed for SAP as described previously (Pilling et al., 2003; Pilling et al., 2007), with the exception that the ELISA plates were coated overnight at 4°C with mouse antihuman SAP antibody (SAP-5; Sigma, St. Louis, MO, USA) diluted 1:1,000 in phosphate-buffered saline (PBS) instead of 50 mM sodium carbonate buffer, and undiluted day 3 conditioned media were assayed.

2.2.5 Staining PBMC with CD86 or MHC Class II

Human PBMCs were cultured in the presence or absence of 8.9 μg/mL TLR3 agonist Poly (I:C), 0.89 μg/mL TLR7 agonist imiquimod (IMIQ), or 2.0 μg/ml nucleotide oligomerization domain (NOD)-like receptor (NLR) agonist peptidoglycan (PGN) in 1 ml in the well of a 48-well plate. On day 1 or 3, 900 μl of the conditioned media were carefully pipetted out and transferred into an Eppendorf tube, snap-frozen in liquid nitrogen and stored at -20°C. A quantity of 500 μl of ice-cold 50 mM ethylenediaminetetraacetic acid (EDTA) in PBS was then added to the PBMCs for 5 minutes at 4°C. The cells were vigorously resuspended with a plastic transfer pipette. The cells were transferred into an Eppendorf tube, collected by centrifugation at 300 × *g* for 5 min and the supernatant was discarded. The remaining cells on the plates were washed with

1 ml of ice-cold PBS, and this solution was added to the first pellet of cells. The cells were again collected by centrifugation at 300 × *g* for 5 min and then resuspended in 200 µl of 4% bovine serum albumin (BSA) in PBS. Cells treated with TLR agonists were divided into two separate Eppendorf tubes. The cells were incubated with 5 µg/ml antihuman CD86 or 5 µg/ml antihuman Human leukocyte antigen-DR/DP/DQ (HLA-DR/DP/DQ) (MHC class II) (both from BD Biosciences) for 30 min at 4°C as described previously (Pilling et al., 2009). Meanwhile, untreated cells were divided into three separate Eppendorf tubes. These cells were incubated with 5 µg/ml antihuman CD86 or 5 µg/ml antihuman HLA-DR/DP/DQ, or they were kept in 4% BSA in PBS for 30 min at 4°C. All of the cells were washed three times in 1 ml of ice-cold PBS and then incubated with 2.5 µg/ml goat antimouse FITC (Southern Biotechnology, Birmingham, AL, USA) for 30 min at 4°C as described previously (Pilling et al., 2009). After washing the cells three times in 1 ml of ice-cold PBS, the cells were resuspended in 100 µl of 4% BSA-PBS, and the staining was analyzed using flow cytometry with an Accuri C6 cytometer (Accuri Cytometers Inc.).

2.2.6 Detection of Ig molecules by Western blot analysis

Human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was diluted to 10 µg/ml, 1 µg/ml and 0.1 µg/ml in PBS. A quantity of 10 µl of conditioned media from day 3 or diluted human IgG was mixed with 2.5 µl of sodium dodecyl sulfate (SDS) sample buffer containing 20 mM dithiothreitol

(DTT) and heated to 100°C for 5 min. After electrophoresis of the samples on 4-15% Tris-glycine polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA), proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) in Tris-NaCl-SDS buffer containing 20% methanol. Western blot staining was performed as described previously (Pilling et al., 2003), with the exception that the detection antibody was 0.05 μg/ml biotinylated goat Fab′(2) antihuman Ig (H+L) (Southern Biotechnology) followed by 1:5,000 ExtrAvidin-Peroxidase staining (Sigma).

2.2.7 Treating monocytes with different combinations of cytokines

After analyzing the day 3 conditioned media with multi-analyte ELISArray kits, we calculated the concentrations of IL-6, IFN-γ, TNF-α, G-CSF, and TGF-β1 in LTA (TLR2 agonist)-treated conditioned media, LPS (TLR4 agonist)-treated conditioned media and control conditioned media. Cocktails of cytokines corresponding to twice the observed concentrations were made in SFM. A quantity of 100 μl of purified human monocytes at a concentration of 5×10^5 cells/ml were incubated in 100 μl of SFM, 100 μl of the above cytokine cocktails or 100 μl of the cytokine cocktail with all five of the above cytokines at either 2,000 pg/ml, 1,000 pg/ml, 200 pg/ml or 20 pg/ml. After 5 days, cells were fixed and stained, and fibrocytes were counted.

2.2.8 Statistics

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Statistical significance was determined using either anyalysis variance (ANOVA) or a *t-test,* and significance was defined as $P < 0.05$.

2.3 Results

2.3.1 *TLR3, TLR4, TLR5, TLR7, TLR8 and TLR9 agonists do not inhibit the differentiation of PBMCs to fibrocytes*

To investigate the role of TLR agonists on fibrocyte differentiation, human PBMCs were cultured in the presence of various TLR agonists. Since immune cells can affect each other, we used PBMCs instead of purified monocytes to more closely mimic a human immune system. All of the TLR agonists were reconstituted in endotoxin-free water, and a control series of water dilutions had no discernible effect on fibrocyte differentiation (data not shown).

All TLR agonists were used in dose ranges that had induced responses in previous studies (Table 2.3.1). TLR3, TLR7, TLR8 and TLR9 agonists consisted of viral, bacterial or synthetic nucleic acids that stimulated their corresponding receptors. When added to PBMCs, none of these agonists showed any significant effect on fibrocyte differentiation (Figures 2.3.1 and 2.3.2). The TLR9 agonist *Escherichia coli* ssDNA appears to decrease fibrocyte differentiation at 10 μg/ml (Figure 2.3.2B), but it was not significant by either ANOVA or *t*-test. One batch of the TLR7 agonist IMIQ inhibited fibrocyte differentiation at 3.3 μg/ml, but other batches of IMIQ from the same manufacturer had no significant effect on fibrocyte differentiation. Although TLR3, TLR7, TLR8 and TLR9 agonists do not affect fibrocyte differentiation, the TLR8 agonist ssRNA and the TLR9 agonist ODN2006 that we used for these studies induced extracellular TNF-α

accumulation by PBMCs (Figure 2.3.3A). TLR3 and TLR7 agonists do not significantly enhance the production of TNF-α, but the TLR3 agonist Poly (I:C) significantly increased the number of CD86-positive cells at day 1 (Figure 2.3.3B) and the TLR7 agonist IMIQ significantly increased the number of HLA-DR/P/Q (MHC class II)-positive cells at day 3 (Figure 2.3.3C), which suggest that these agonists still have biological effects on PBMCs.

Table 2.3.1. TLR agonists stimulate different cytokines and effectors in various cell types[a]

Agonist	TLR	Concentration used	Cell type	Effect	References
Pam3CSK4 (synthetic)	TLR2	0.30 μg/ml	PBMC	Induction of IL-6	(Taylor et al., 2006)
Lipomannan *M. smegmatis* (LM-MS)	TLR2	10 μg/ml	PBMC	Cellular aggregation	(Puissegur et al., 2007)
Heat-killed *Listeria monocytogenes* (HKLM)	TLR2	3 x 10^7 cells/ml	PBMC	Induction of type I and type II IFN	(Ghosh et al., 2006)
Lipotechoic acid from *S. aureus* (LTA)	TLR2	10 μg/ml	RAW264.7	Increased expression of IL-1b, TNF-α, IL-6, and IP-10	(Paulnock et al., 2000)
Lipopolysaccharide from *P. gingivalis* (PG-LPS)	TLR2	0.1 μg/ml	U373	Secretion of IL-6	(Yoshimura et al., 2002)
FSL-1 (synthetic)	TLR2	0.1 μg/ml	PBMC	Induction of Type I and Type II IFN	(Ghosh et al., 2006)
Poly (I:C) (synthetic)	TLR3	10 μg/ml	PBMC	Production of IL-8, MCP-1, and TNF-α	(Uehara et al., 2007)
E. coli K12 lipopolysaccharide (LPS)	TLR4	0.01, 0.1, and 1 μg/ml	PBMC	Production of TNF-α, IL-6, and IL-10	(Bekeredjian-Ding et al., 2006; Wang et al., 2000)
S. typhimurium flagellin	TLR5	0.1 μg/ml	PBMC	Activation of NF-κB	(Zeng et al., 2006)
Imiquimod (IMIQ) (synthetic)	TLR7	3 μg/ml	PBMC	Expression of IFN, TNF-α, IL-6, and IL-8 increased	(Megyeri et al., 1995)
Gardiquimod (GIMIQ) (synthetic)	TLR7	1 μg/ml	Plasmacytoid dendritic cells	Expression of IFN-α, IFN-β, and RANTES increased	(Phipps-Yonas et al., 2008)

Loxoribine (synthetic)	TLR7	0.2 mM	RAW264.7 mouse macrophage cell line NK cells	Expression of IL-23 p19 increased Activation of NK cells	(Al-Salleeh and Petro, 2007; Pope et al., 1993)
ssRNA40 (synthetic)	TLR8	1 μg/ml	Peripheral blood	Activation of TNF-α	(Levy et al., 2006)
E. coli ssDNA/LyoVec	TLR9	0.3-10 μg/ml	RAW264.7	Production of nitrite	(Roberts et al., 2005)
ODN2006 (synthetic)	TLR9	2 μM	PBMC	Increased production of IFN-γ, IFN-α, IL-6, IL-8, and IL-12	(Bauer et al., 1999; Bauer et al., 2001)
ODN2216 (synthetic)	TLR-9	1 μM	Plasmacytoid dendritic cells	Decreased production of IFN-α	(Kwok et al., 2008)
Peptidoglycan from *S. aureus* (PGN)	NLR	0.1-10 μg/ml	HEK-293	Activation of NF-κB	(Mitsuzawa et al., 2001)

[a]This table summarizes the concentration of agonists used by other workers. TLR, Toll-like receptor; PBMC, human peripheral blood mononuclear cells; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; NF-κB, nuclear factor-κB; HEK-293, human embryonic kidney-293 cells; PGN, peptidoglycan.

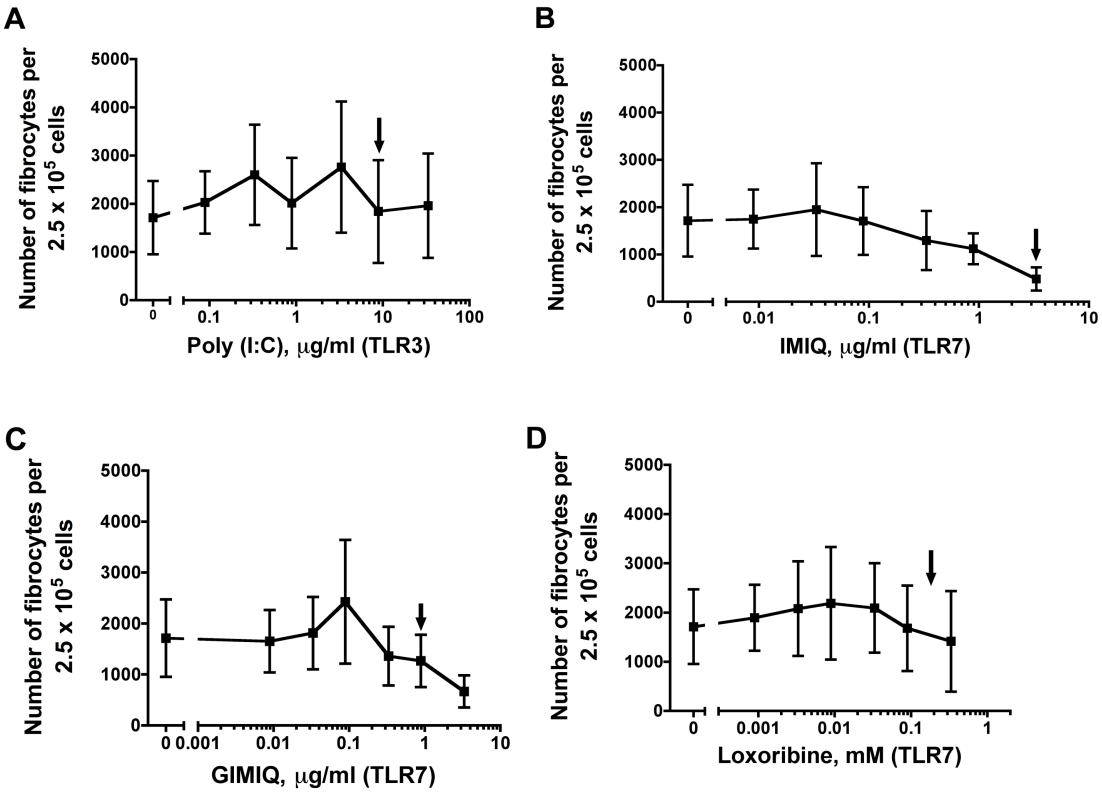


Figure 2.3.1: Toll-like receptor 3 (TLR3) and TLR7 agonists do not affect fibrocyte differentiation. Human peripheral blood mononuclear cells (PBMCs) were cultured in different concentrations of the TLR3 agonist polyinosine-polycytidylic acid (Poly I:C) and the TLR7 agonists imiquimod (IMIQ), gardiquimod (GIMIQ) and loxoribine. After 5 days, the cells were air-dried, fixed and stained, and the number of fibrocytes was counted. The results are means ± SEM of fibrocytes per 2.5×10^5 PBMCs (n = 3 separate experiments). Although high concentrations of IMIQ and GIMIQ appear to decrease the number of fibrocytes compared to no addition, the decrease is not significant by either

analysis of variance (ANOVA) or *t*-test. Arrows indicate the reported concentrations for the TLR agonists previously shown to be functional (Table 2.3.1).

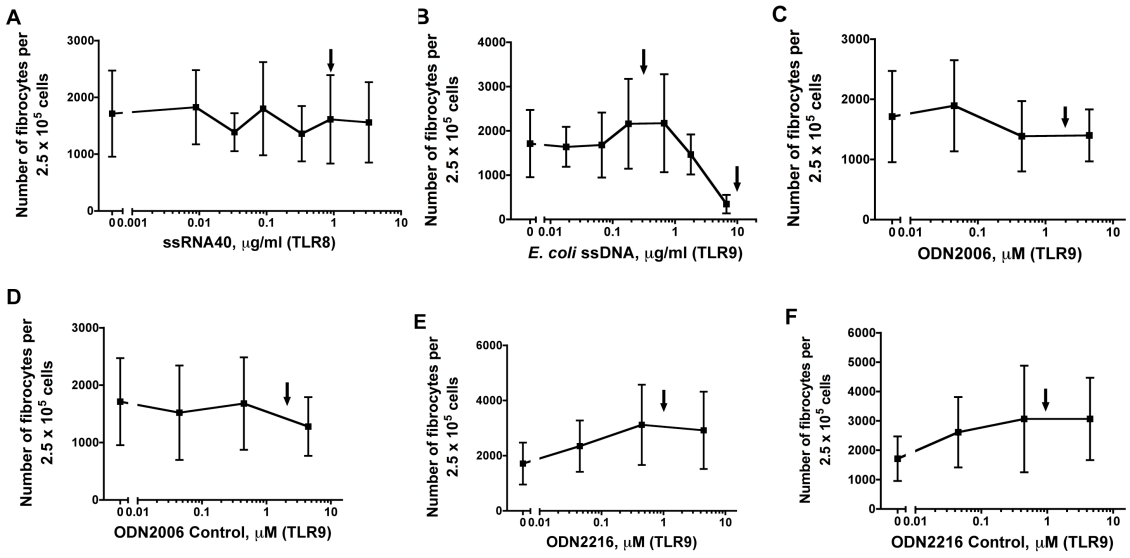


Figure 2.3.2 TLR8 and TLR9 agonists do not affect fibrocyte differentiation. Human PBMCs were cultured in different concentrations of the TLR8 agonist single-stranded RNA40 (ssRNA40) and the TLR9 agonists *Escherichia coli* ssDNA, ODN2006 and ODN2216. ODN2006 and ODN2216 are synthetic oligonucleotides that contain CpG dinucleotides at specific sequences. The controls for these oligonucleotides are ODN2006 control and ODN2216 control, which contain GpC dinucleotides instead of CpG dinucleotides. After 5 days, the cells were air-dried, fixed and stained, and the number of fibrocytes was counted. The results are means ± SEM of fibrocytes per 2.5×10^5 PBMCs (n = 3 separate experiments). Although high concentrations of *E. coli* ssDNA appear to decrease the number of fibrocytes compared to no addition, the decrease is not significant

by either ANOVA or *t*-test. High concentrations of ODN2216 and ODN2216 control appear to increase fibrocyte number compared to no addition, but the increase is not significant by either ANOVA or *t*-test. Arrows indicate the concentrations of TLR agonists used by other workers (Table 2.3.1).

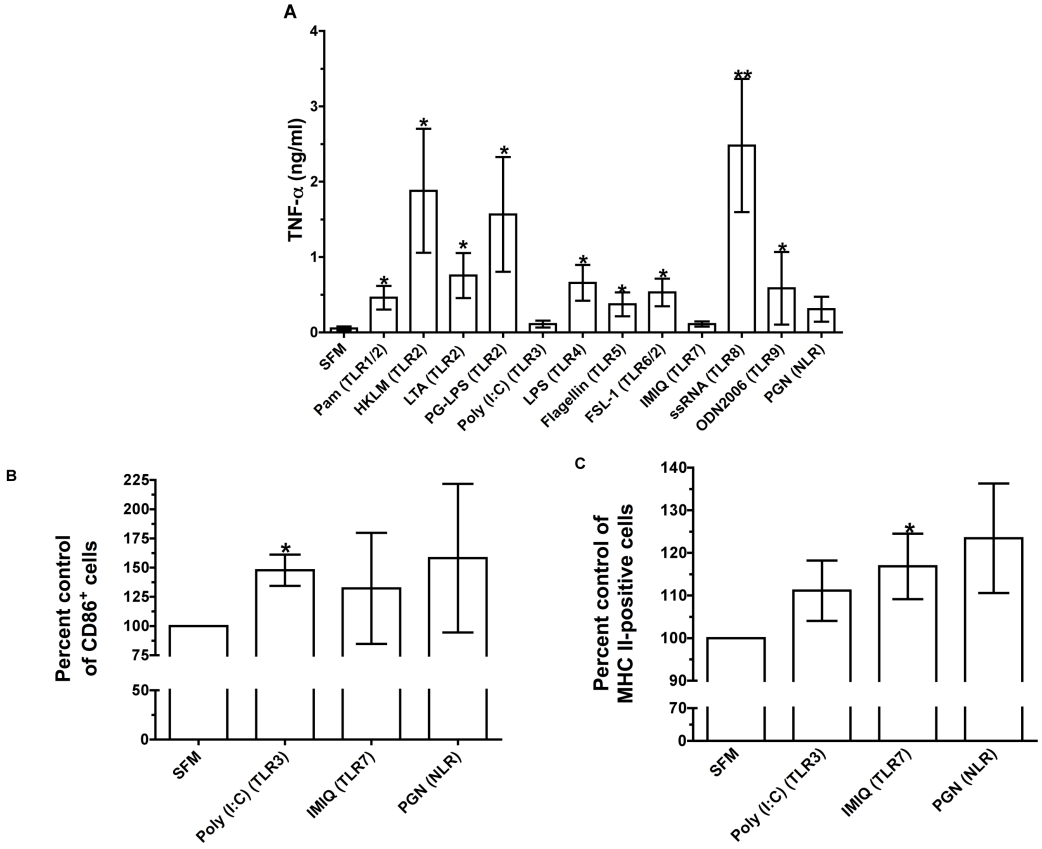


Figure 2.3.3 TLR agonists affect PBMCs. (A). Human PBMCs were cultured in the absence (serum-free medium (SFM) control) or the presence of 0.89 μg/ml TLR2 agonists Pam3CSK4, 8.9×10^7 cells/ml heat-killed *Listeria monocytogenes* (HKLM), 1.79 μg/ml lipotechoic acid (LTA), or 8.9 μg/ml lipopolysaccharides from *Porphyromonas gingivalis* (PG-LPS), 8.9 μg/ml TLR3 agonist Poly (I:C), 0.89 μg/ml TLR4 agonist LPS, 0.89 μg/ml TLR5 agonist flagellin, 0.89 μg/ml TLR2/6 agonist FSL-1, 0.89 μg/ml TLR7 agonist IMIQ, 0.89 μg/ml TLR8 agonist ssRNA, 4.47 μM TLR9 agonist ODN 2006 or 1.79 μg/ml nucleotide oligomerization domain (NOD)-like receptor (NLR) agonist peptidoglycan (PGN). On day 3, the conditioned media were tested for the presence of tumor necrosis factor (TNF)-α

using enzyme-linked immunosorbent assay. The results are means ± SEM of TNF-α concentration (n = 3 or more separate experiments). (B) Human PBMCs were cultured in the presence or absence of Poly (I:C), IMIQ and PGN as described for Figure 2.3.3A. After 1 day, PBMCs were removed and stained for CD86 (n = 3 separate experiments). (C) Human PBMCs were cultured in the presence or absence of 8.9 µg/ml TLR3 agonist Poly (I:C), 0.89 µg/ml TLR7 agonist IMIQ or 1.79 µg/ml NLR agonist PGN. On day 3, PBMCs were removed from the tissue culture plate and stained for major histocompatibility complex (MHC) class II. The results are means ± SEM of positive MHC class II cells (n = 4 separate experiments). *$P < 0.05$ and **$P < 0.01$ compared to no-agonist control (SFM) according to *t*-test.

TLR4 recognizes bacterial LPSs, and TLR5 recognizes bacterial flagellin (Bekeredjian-Ding et al., 2006; Wang et al., 2000; Zeng et al., 2006). We observed that 0.89 μg/ml LPS or 0.89 μg/ml flagellin had no effect on fibrocyte differentiation (Figure 2.3.4). Although the number of fibrocytes decreased in PBMCs treated with LPS or flagellin, *t*-tests showed no significant difference compared to no TLR agonist. Both LPS and flagellin caused PBMCs to increase their accumulation of extracellular TNF-α (Figure 2.3.3A), which suggests that the LPS and flagellin we used have biological effects on PBMCs. Together, the data suggest that TLR3, TLR4, TLR5, TLR7, TLR8 and TLR9 agonists do not significantly affect the *in vitro* differentiation of human fibrocytes.

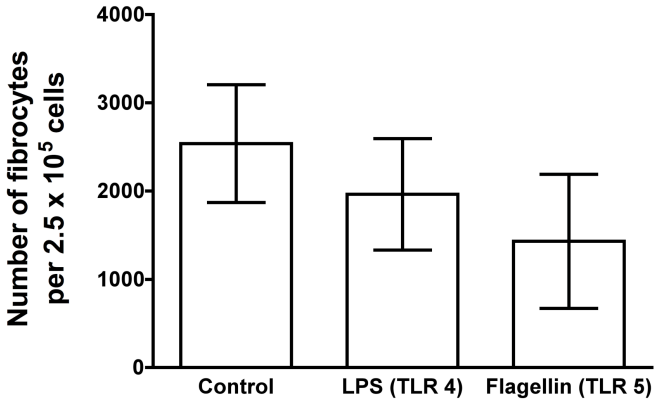


Figure 2.3.4. TLR4 and TLR5 agonists do not affect fibrocyte differentiation. Human PBMCs were cultured in the presence or absence of 0.89 μg/ml TLR4 agonist LPS or 0.89 μg/ml TLR5 agonist flagellin. After 5 days, the cells were air-dried, fixed and stained, and the number of fibrocytes was counted. The results are means ± SEM of fibrocytes per 2.5×10^5 PBMCs (n = 6 separate experiments). Although LPS and flagellin appear to decrease the number of fibrocytes compared to no addition, the decrease is not significant by either ANOVA or *t*-test.

2.3.2 TLR2 agonists inhibit the differentiation of PBMCs to fibrocytes

TLR2 receptors recognize bacterial lipoproteins and lipotechoic acid from bacterial membranes, and they mediate the activation of transcription factors such as NF-κB and IFN-regulatory factors (Gallucci and Matzinger, 2001; Iwasaki and Medzhitov, 2004; Janeway and Medzhitov, 1999; Trinchieri and Sher, 2007). We found that at concentrations similar to those used in other studies (Table 2.3.1), all six TLR2 agonists tested inhibited fibrocyte differentiation (Figure 2.3.5). At day 5, the TLR2 agonist-treated cells appeared to be viable as determined by phase contrast microscopy (Figure 2.3.6). At day 5 after TLR2 agonist treatment, there was no significant difference in the number of viable PBMCs treated with or without TLR2 agonists (Figure 2.3.6B).

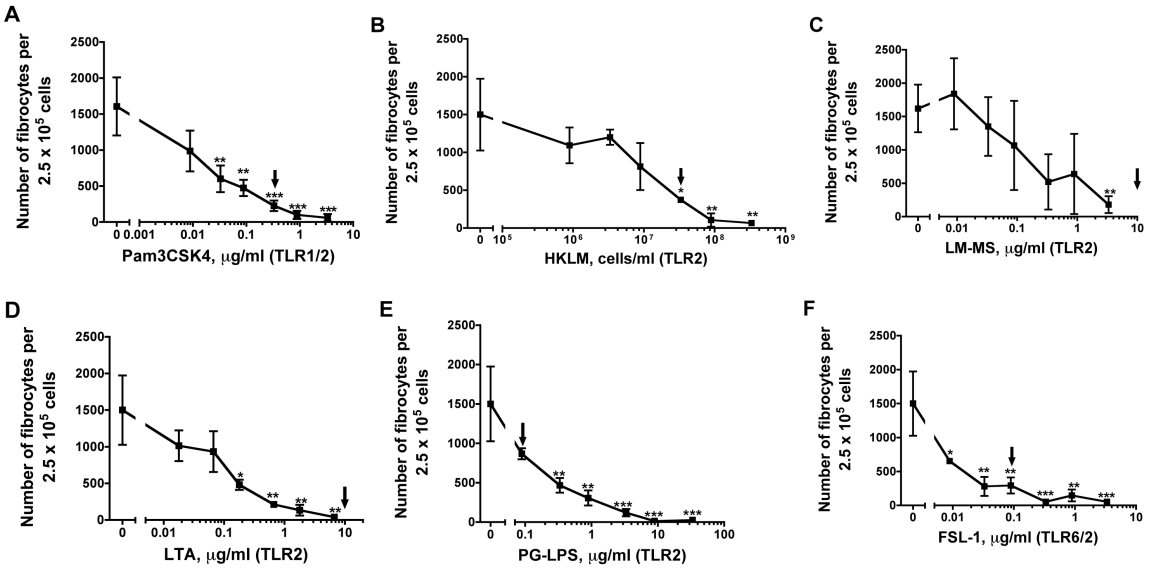


Figure 2.3.5. TLR2 agonists inhibit fibrocyte differentiation. Human PBMCs were cultured in different concentrations of the TLR2 agonists Pam3CSK4, HKLM, lipomannans from *Mycobacterium smegmatis* (LM-MS), LTA, PG-LPS and FSL-1. After 5 days, the cells were air-dried, fixed and stained, and the number of fibrocytes was counted. The results are means ± SEM of fibrocytes per 2.5×10^5 PBMCs (n = 6 separate experiments for Pam3CSK4 and n = 3 separate experiments for the rest of the TLR2 agonists). The absence of error bars indicates that the error was smaller than the plot symbol. Statistical significance was analyzed by ANOVA using Dunnett's test to compare values against the no-agonist control for all the agonists except LM-MS, which was analyzed by *t*-test.

*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared to no-agonist control. Arrows indicate the concentration of TLR2 agonists used by other workers (Table 2.3.1).

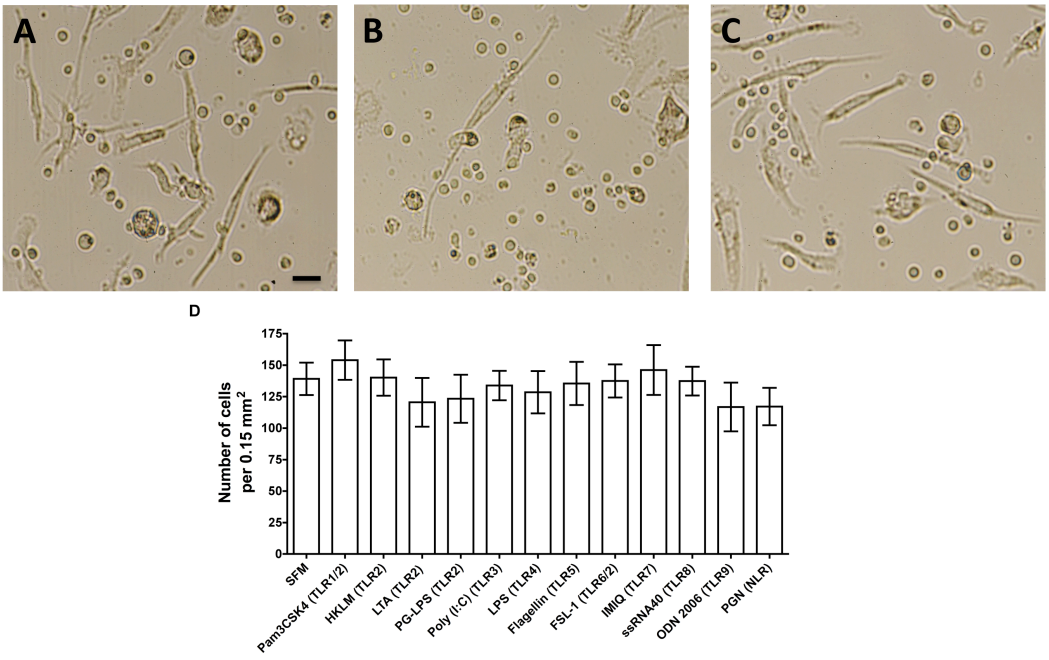


Figure 2.3.6. Human PBMCs cultured in the presence of TLR2 agonists are viable. Human PBMCs were cultured in the (A) absence or (B) presence of 1.79 µg/ml TLR2 agonist LTA or (C) 0.89 µg/ml TLR7 agonist IMIQ. After 5 days, the cells cultured in different conditions were photographed. Bar is 20 µm. (D) Human PBMCs were cultured in the absence (SFM control) or presence of 0.89 µg/ml TLR2 agonists Pam3CSK4, 8.9×10^7 cells/ml HKLM, 0.89 µg/ml LM-MS, 1.79 µg/ml LTA, 8.9 µg/ml PG-LPS or 0.89 µg/ml FSL-1 for 5 days. After 5 days, the cells were photographed and the number of cells per picture was counted. The results are means ± SEM of cells per 0.15 mm^2 (n = 3 separate experiments).

Besides TLRs, there are also other pattern recognition receptors (PRRs) present in PBMCs that detect a variety of microbial molecules, including NLRs, that contain a NOD- and ligand-recognizing leucine-rich repeat (Philpott and Girardin, 2004). NLRs are intracellular receptors that recognize a variety of microbial molecules. We investigated the role of PGN on fibrocyte differentiation, since this agonist appears to be a NLR agonist (Travassos et al., 2004). When added to PBMCs, PGN at concentrations from 0.01 to 6.7 μg/ml did not affect fibrocyte differentiation (Figure 2.3.7). Although not statistically significant, PGN increased the number of CD86- and MHC class II-positive cells (Figures 2.3.3B and 2.3.3C). Together, the data suggest that although PGN has no effect on fibrocyte differentiation when added to PBMCs, it still has some effect on cells.

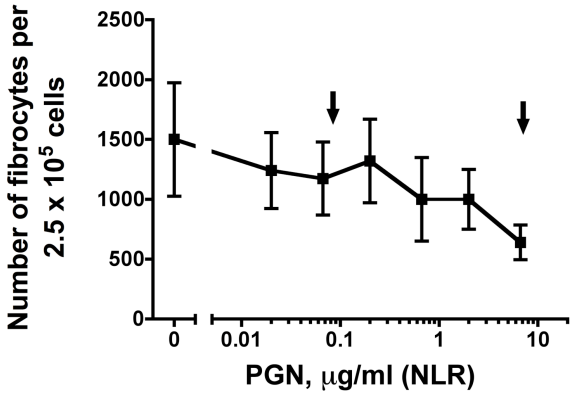


Figure 2.3.7. The NLR agonist PGN does not affect fibrocyte differentiation. Human PBMCs were cultured in different concentrations of PGN. After 5 days, the cells were air-dried, fixed and stained, and the number of fibrocytes was counted. The results are means ± SEM of fibrocytes per 2.5×10^5 PBMCs (n = 3 separate experiments). There was no statistically significant difference between the effect of any PGN concentration and control, according to ANOVA or *t*-test. Arrows indicate the concentrations of PGN used by other workers (Table 2.3.1).

2.3.3 Some TLR2 agonists inhibit fibrocyte differentiation indirectly

To determine whether TLR2 agonists act directly on human monocytes, we examined the effect of TLR2 agonists on purified monocytes. When TLR2 agonists were added to purified human monocytes, the TLR2 agonists did not inhibit the differentiation of monocytes to fibrocytes (Figure 2.3.8). This suggests that TLR2 agonists do not inhibit fibrocyte differentiation by acting directly on monocytes. Interestingly, the NLR agonist PGN potentiated the differentiation of monocytes to fibrocytes (Figure 2.3.8). Since PGN had little activity when added to PBMCs (Figure 2.3.7), PGN might act directly on monocytes to potentiate fibrocyte differentiation and this activity might be inhibited by the presence of the other cells in the PBMC population. TLR4 agonist LPS also potentiated the differentiation of monocytes to fibrocytes (Figure 2.3.8), though the increase was not significant by either ANOVA or *t*-test.

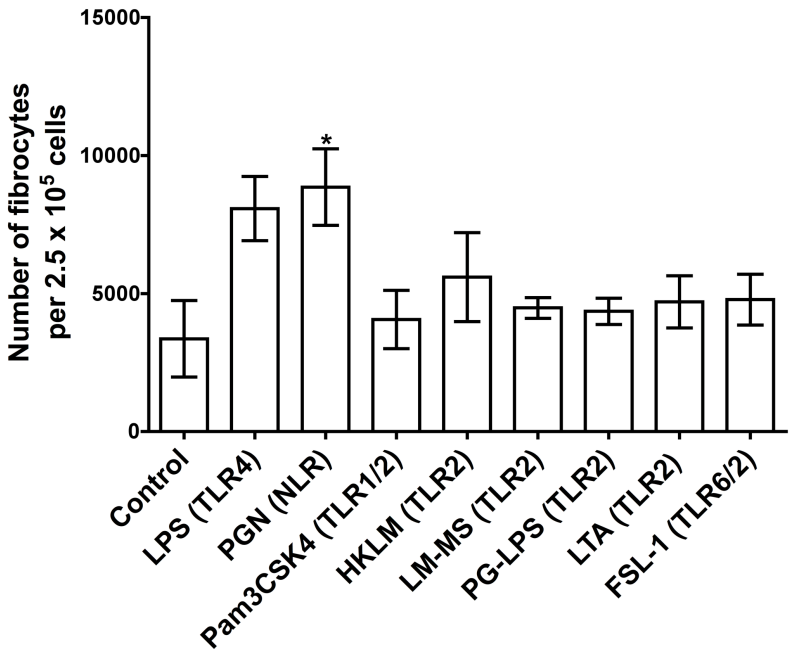


Figure 2.3.8. TLR2 agonists do not inhibit the differentiation of purified monocytes to fibrocytes. Human monocytes were cultured in SFM for 5 days in the presence of LPS (1 μg/ml), PGN (2 μg/ml), Pam3CSK4 (1 μg/ml), HKLM (1 x 10^8 cells/ml), LM-MS (1 μg/ml), PG-LPS (10 μg/ml), LTA (2 μg/ml) and FSL-1 (1 μg/ml). The results are means ± SEM of fibrocytes per 2.5 × 10^5 PBMCs (n = 4 separate experiments). *P < 0.05 compared to control (one-way ANOVA, Dunnett's test).

Since TLR2 agonists do not act directly on monocytes, a possible explanation for the ability of TLR2 agonists to inhibit fibrocyte differentiation when added to PBMCs is that the TLR2 agonists cause some cells in the PBMC population, such as T, B or NK cells, to secrete a factor that inhibits the differentiation of monocytes to fibrocytes. To test this possibility, we incubated PBMCs for 3 days with TLR2 agonists, collected the conditioned media and then added this to monocytes from the same donor. As previously observed, when added to PBMCs, TLR2 agonists inhibited the differentiation of fibrocytes (Figure 2.3.9A). Conditioned media from PBMCs incubated with the TLR2 agonists lipomannans from *Mycobacterium smegmatis* (LM-MS), LPS from *Porphyromonas gingivalis* (PG-LPS) and LTA for 3 days inhibited the differentiation of monocytes to fibrocytes (Figure 2.3.9B). This suggests that these TLR2 agonists cause some nonmonocyte cells in the PBMC population to increase the accumulation of fibrocyte-inhibiting factors or to decrease the accumulation of fibrocyte-promoting factors in the extracellular medium.

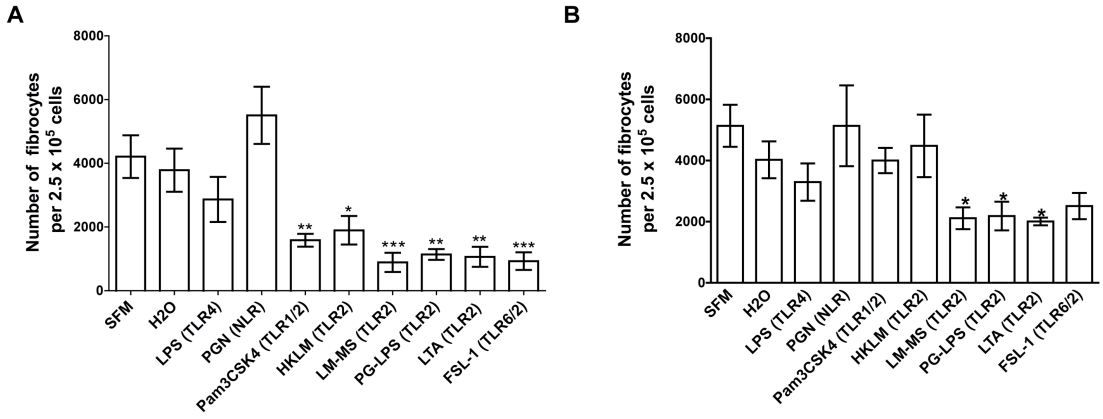


Figure 2.3.9. Conditioned media from PBMCs incubated with LM-MS, PG-LPS and LTA inhibit fibrocyte differentiation. (A) Human PBMCs were cultured for 5 days in the presence of the TLR2 agonists Pam3CSK4, HKLM, LM-MS, PG-LPS, LTA and FSL-1, the TLR4 agonist LPS, and the NLR agonist PGN, using the same concentrations as in Figure 2.3.6. SFM was used as a buffer control, and LPS and PGN were used as negative controls, since these agonists do not inhibit fibrocyte differentiation. Conditioned media were removed on day 3 from duplicates of these wells. The results are means ± SEM of fibrocytes per 2.5 × 10^5 PBMCs (n = 5 separate experiments). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared to control (one-way ANOVA, Dunnett's test). (B) Human monocytes were cultured for 5 days in the presence of the conditioned media from day 3

PBMCs incubated with TLR2 agonists. The results are means ± SEM of fibrocytes per 2.5×10^5 PBMCs (n = 5 separate experiments). Conditioned media from PBMCs incubated with the TLR2 agonists LM-MS, PG-LPS and LTA significantly inhibited the differentiation of monocytes to fibrocytes. $^*P < 0.05$ compared to control (one-way ANOVA, Dunnett's test).

2.3.4 Some TLR2 agonists cause PBMCs to secrete an unknown factor that inhibits fibrocyte differentiation

We previously found that 1 ng/ml or more IFN-γ, 0.5 μg/ml or more SAP or 10 μg/ml or more aggregated IgG directly inhibits monocytes from differentiating into fibrocytes and that 1 ng/ml or more IL-12 indirectly inhibits fibrocyte differentiation ((Pilling et al., 2003; Pilling et al., 2006; Shao et al., 2008). In cultures of PBMCs containing fetal bovine serum, IFN-α inhibits fibrocyte differentiation (Wang et al., 2007a). In our serum-free culture system, 0.1 ng/ml or more IFN-α also inhibited fibrocyte differentiation (Figure 2.3.10). To determine whether one of these inhibitory factors is the antifibrocyte factor present in conditioned media from PBMCs incubated with the TLR2 agonists LM-MS, PG-LPS and LTA, we examined the levels of IFN-α, IFN-γ, IL-12, SAP and IgG in the conditioned media. Conditioned media from PBMCs incubated with TLR2 agonists as well as the TLR4 agonist LPS did not show any detectable levels of IL-12 or SAP, with a detection limit of 0.1 ng/ml for both factors (data not shown). These conditioned media also had less than 0.01 ng/ml IFN-γ (Table 2.3.2 and data not shown). The conditioned media also did not show any detectable levels of either IFN-α or IgG, with a detection limit of 1 pg/ml IFN-α and 1 μg/ml IgG (data not shown). We further analyzed day 3 conditioned media from PBMCs incubated with Pam3CSK4, PG-LPS, LTA, PGN and LPS for the presence of Th1/Th2/Th17 cytokines. There were no detectable levels of IL-2, IL-4, IL-5, IL-10, IL-12, IL-13 or IL-17A with a detection level of approximately 15 pg/ml for

each of the cytokines. We detected IL-6, TNF-α, IFN-γ, G-CSF and TGF-β1 in conditioned media from LTA (TLR2 agonist)-treated PBMCs, LPS (TLR4 agonist)-treated PBMCs, and control PBMCs (Table 2.3.2). We then analyzed the effect of cocktails of these five cytokines on fibrocyte differentiation. Cytokine cocktails corresponding to the amount of the five cytokines found in LTA-treated conditioned media, LPS-treated conditioned media or control conditioned media did not have any significant effect on fibrocyte differentiation (Figure 2.3.11). In addition, cocktails containing higher levels of the five cytokines had no significant effect on fibrocyte differentiation (Figure 2.3.11). These observations indicate that the LTA-induced factor in the PBMC conditioned media that inhibits fibrocyte differentiation is not one of the five cytokines that we found in LTA-treated conditioned media and thus appears to be a novel factor.

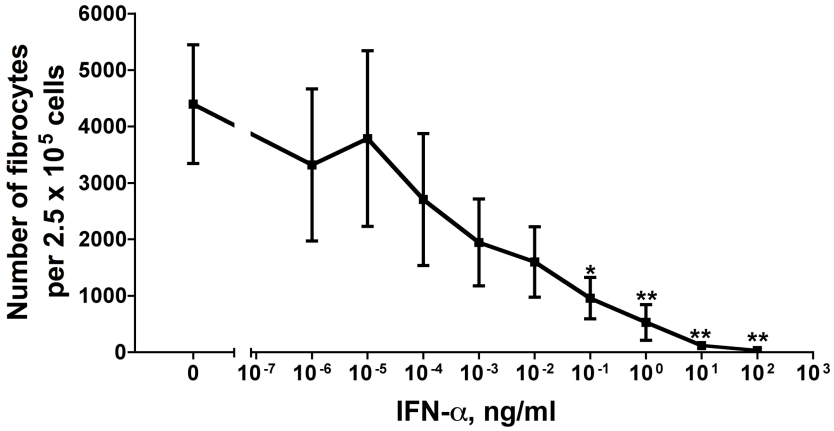


Figure 2.3.10. Interferon (IFN)-α inhibits fibrocyte differentiation. Human PBMCs were cultured in different concentrations of IFN-α. After 5 days, the cells were air-dried, fixed and stained, and the number of fibrocytes was counted. The results are means ± SEM of fibrocytes per 2.5×10^5 PBMCs (n = 3 separate experiments). *$P < 0.05$ and **$P < 0.01$ compared to no IFN-α control (one-way ANOVA, Dunnett's test).

Table 2.3.2 Conditioned media from LTA-treated PBMC, LPS-treated PBMC, and control conditioned media contain low levels of IL-6, TNF-α, IFN-γ, G-CSF and TGF-β1*

Cytokine	Concentration in control CM (pg/ml)	Concentration in LPS-treated CM (pg/ml)	Concentration in LTA-treated CM (pg/ml)
IL-6	4 ± 12	530 ± 250	750 ± 250
TNF-α	4.6 ± 1.6	29 ± 13	33 ± 6
IFN-γ	1.0 ± 0.5	2.4 ± 0.9	4.5 ± 3.5
G-CSF	2.5 ± 0	17 ± 0	9.3 ± 1.8
TGF-β1	13 ± 1.8	3.5 ± 0	4.5 ± 0

*The above five cytokines were detected in conditioned media (CM) from LTA-treated PBMCs, LPS-treated PBMCs and control PBMCs. This assay measures total transforming growth factor (TGF)-β1 as opposed to active TGF-β1. The results are means ± SEM of cytokine concentration (n = 2 separate experiments).

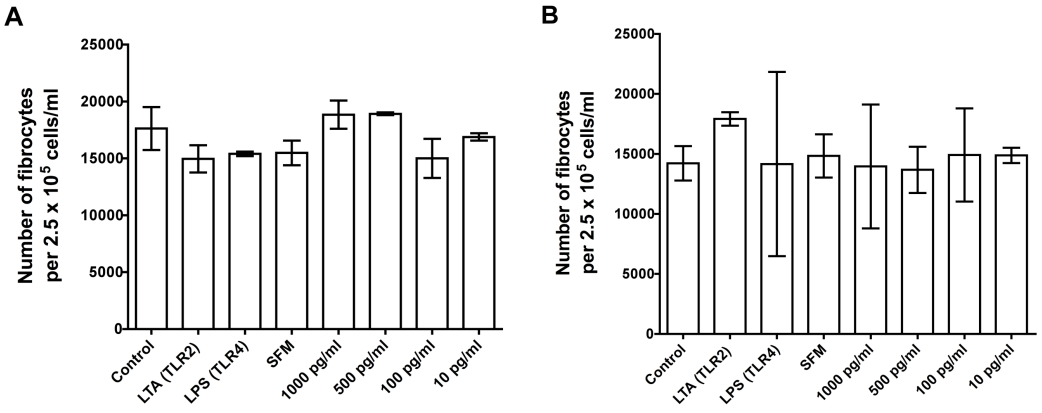


Figure 2.3.11. Cytokine mixtures from SFM, LTA or LPS conditioned media had no effect on fibrocyte differentiation. Human PBMCs were cultured in the absence of added cytokines (control) or in mixtures of cytokines (IL-6, TNF-α, granulocyte colony-stimulating factor (G-CSF), transforming growth factor (TGF)-β1, and IFN-γ) at the concentrations found in conditioned media from SFM-, LTA-, or LPS-treated PBMCs. Human PBMCs were also cultured in the presence of mixtures containing 1000 pg/ml, 500 pg/ml, 100 pg/ml, or 10 pg/ml of each of the five cytokines. After 5 days, the cells were dried, fixed and stained, and the number of fibrocytes was counted. The results are means ± SEM of fibrocytes per 2.5 × 10^5 PBMCs. (A) Results from one donor. (B) Results from a second

donor. For each donor, there was no significant difference between any cytokine mixture and the control (1-way ANOVA, Dunnett's test and *t*-test).

2.4 Discussion

We found that a variety of TLR2 agonists inhibit fibrocyte differentiation. However, the TLR2 agonists do not directly inhibit the differentiation of monocytes to fibrocytes, but rather cause some other cell type in the PBMC population to inhibit monocytes from differentiating into fibrocytes. There are five known factors (IFN-α, IFN-γ, IL-12, SAP and aggregated IgG) which inhibit fibrocyte differentiation (Pilling et al., 2003; Pilling et al., 2006; Shao et al., 2008; Wang et al., 2007a). Our data suggest that PBMCs incubated with some TLR2 agonists secrete an unknown sixth factor that inhibits fibrocyte differentiation.

The TLR2 agonist LTA induces PBMCs to increase the extracellular accumulation of IL-6, TNF-α, IFN-γ and G-CSF and to decrease the extracellular accumulation of TGF-β1. Both TLR2 agonists and TLR4 agonist induce primary adrenocortical cells to secrete IL-6 (Kanczkowski et al., 2009; Taylor et al., 2006). However, we previously found that IL-6 has no effect on fibrocyte differentiation (Shao et al., 2008). Like the above five factors, this combination of cytokines at the concentrations found in the conditioned media, or at higher concentrations, was unable to inhibit fibrocyte differentiation and is thus not the factor in LTA-stimulated conditioned medium that inhibits fibrocyte differentiation.

Monocytes express TLR1-TLR9, but not TLR10 (Hornung et al., 2002; Zarember and Godowski, 2002). They have a high expression of TLR1, TLR2 and TLR4; intermediate expression of TLR 5, TLR6 and TLR8; and low expression of TLR7 and TLR9 (Hornung et al., 2002; Zarember and Godowski, 2002). Once activated, TLR agonists trigger downstream signaling events by one of four adaptor molecules: MyD88, MyD88-like adaptor protein (Mal), Toll/Interleukin-1 receptor domain-containing adaptor protein-inducing IFN-β or TRIF-related adaptor molecule (TRAM) (Akira, 2006; Farhat et al., 2008; Hoebe et al., 2004; Keating et al., 2007). MyD88 activates mitogen-activated protein kinases (MAPKs) through IL-1R-associated kinase (MAPKs) and tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF-6) (Akira, 2006; Farhat et al., 2008). The signaling eventually causes the translocation of transcription factors such as NF-κB and activator protein 1 (AP-1), which then induces the production of inflammatory cytokines such as TNF-α, IL-6, IL-1β and IL-12 (Farhat et al., 2008). TLR3 does not require the MyD88 pathway; instead it induces the production of IFN-β via TRIF (Farhat et al., 2008; Yamamoto et al., 2003). TLR4 can cause NF-κB activation by either a MyD88-dependent pathway or a MyD88-independent pathway (Keating et al., 2007). TLR4 activates a MyD88-independent pathway via TRIF, which complexes with TRAM and leads to NF-κB activation (Keating et al., 2007). Since none of the TLR agonists appeared to directly inhibit the differentiation of monocytes to fibrocytes, our data suggest that

none of the above signal transduction pathways in monocytes affects their ability to differentiate into fibrocytes.

TLR2 recognizes various bacterial components such as bacterial lipoproteins and LTA (Farhat et al., 2008; Gerold et al., 2008). PBMCs incubated with the TLR2 agonists LM-MS, PG-LPS and LTA secrete an unknown factor that inhibits fibrocyte differentiation. However, for unknown reasons, the TLR2 agonists Pam3CSK4 (a synthetic triacylated lipopeptide), heat-killed *Listeria monocytogenes* (HKLM) and FSL-1 (a synthetic lipoprotein) do not appear to cause other cells in the PBMC population to secrete factors that inhibit fibrocyte differentiation. This suggests that these agonists indirectly inhibit fibrocyte differentiation by either cell-cell contact or a labile secreted factor.

We previously found that aggregated IgG inhibits fibrocyte differentiation (Pilling et al., 2006). We hypothesized that since aggregated IgG (that is, IgG bound to something such as a bacterium) signifies the presence of bacterial infection, and since closing an infected wound and thus forming an anaerobic pocket can be detrimental, the inhibition of fibrocyte differentiation by aggregated IgG might have evolved to prevent closure of septic wounds by fibrocytes. An interesting possibility is that the indirect inhibition of fibrocyte differentiation by TLR2 agonists allows other cells in a wound environment to check for the presence of bacteria, and if bacteria are present, to relay this information to

monocytes and prevent fibrocyte differentiation and the possible closure of an infected wound.

Chapter 3: High and low molecular weight hyaluronic acid differentially regulate human fibrocyte differentiation

3.1 Introduction

During tissue injury, the extracellular matrix component hyaluronic acid (HA) breaks down into smaller fragments (Jiang et al., 2007; Li et al., 2011; McKee et al., 1996). HA is a negatively charged linear polymer of repeating units of (β,1-4)-D-glucuronic acid-(β,1-3)-N-acetyl-D-glucosamine that gives mechanical strength to tissues (Laurent and Fraser, 1992). High molecular weight hyaluronic acid (HMWHA) has a molecular mass > 1 x 10^6 Da and is found in normal healthy tissue (Teder et al., 2002). The concentration of hyaluronic acid is 15-150 μg/g in lung tissue, 200 μg/g in the vitreous humor of the eye, 500 μg/g in skin, and 1400-3600 μg/g in synovial fluid (Kuo, 2005). In injured tissue, HMWHA breaks down to low molecular weight HA (LMWHA) (Jiang et al., 2007). LMWHA masses range from 0.8 to 8 x 10^5 Da (Jiang et al., 2007). However, there are variations in the use of the terms HMWHA or LMWHA. HMWHA often refers to any hyaluronic acid that has not been degraded (Filion and Phillips, 2001), therefore, in this chapter, I will use HMWHA for hyaluronic acid that is greater than 1 x 10^6 Da, LMWHA for 0.8 to 8 x 10^5 Da hyaluronic acid, and oligo-HA for < 6 x 10^3 Da hyaluronic acid. Cells appear to be able to sense the difference between HMWHA, LMWHA, and oligo-HA (Bollyky et al., 2007; Chang et al., 2007; Day and de la Motte, 2005; McKee et

al., 1996; Nakamura et al., 2004; Scheibner et al., 2006; Termeer et al., 2002; Termeer et al., 2000). For instance, LMWHA but not HMWHA stimulates alveolar macrophages to secrete inflammatory cytokines such as IL-8 (McKee et al., 1996), while the maturation and activation of monocyte-derived dendritic cells is promoted by 1.2 x 10^3 Da HA, but not HMWHA or LMWHA (Termeer et al., 2000).

One of the major receptors that monocytes and lymphocytes express to detect HA is CD44 (Jiang et al., 2005; Jiang et al., 2007; Peach et al., 1993; Teder et al., 2002). During lung injury, CD44 is used to clear degraded HA (McKee et al., 1996; Taylor et al., 2007b; Teder et al., 2002). HA-CD44 interactions help the movement of migratory cells during development and help the migration of immune cells into injured sites (Jiang et al., 2007; Laurent and Fraser, 1992; Siegelman et al., 1999). HA-CD44 interactions also promote the adhesion and motility of fibroblasts, thus facilitating tissue repair and remodeling of the injured sites (Svee et al., 1996). Monocytes, dendritic cells, and lymphocytes also bind HA using Toll-like receptors (TLR) such as TLR2 and TLR4 (Scheibner et al., 2006; Termeer et al., 2002). LMWHA binds to either TLR2 or TLR4 to elicit pro-inflammatory action, while HMWHA dampens inflammation by inhibiting TLR2 or TLR4 signaling (Campo et al., 2010; Scheibner et al., 2006). HA can also bind CD168 (receptor for hyaluronan-mediated motility, RHAMM), a cell-surface receptor on fibroblasts and

macrophages (Zaman et al., 2005). RHAMM is upregulated during inflammation and cancer (Hamilton et al., 2007; Zaman et al., 2005). Finally, HA can also bind lymphatic vessel endothelial HA receptor (LYVE), which is found predominately in lymphatic endothelial cells and appears to clear HA from lymph (Johnson et al., 2007).

Since LMWHA increases during tissue injury (Jiang et al., 2007), in this chapter I examined the effect of hyaluronic acid on fibrocyte differentiation. I found that HMWHA promotes fibrocyte differentiation, while LMWHA inhibits fibrocyte differentiation.

3.2 Methods

3.2.1 Culturing PBMC with hyaluronic acid, cytokines, SAP, and antibodies

Peripheral blood mononuclear cells (PBMC) were isolated and incubated in RPMI-based serum free media (SFM) as described in chapter 2.2.1. High molecular weight hyaluronic acid from rooster comb (HMWHA) and low molecular weight hyaluronic acid from umbilical cord (LMWHA) (both from Sigma-Aldrich, St. Louis, MO) were reconstituted to 25 mg/ml in water. 1,230 Da oligo-HA (Hyalose, Oklahoma City, OK) was reconstituted to 10 mg/ml in water. All experiments were performed with at least three different batches of hyaluronic acid. In some experiments, human serum amyloid P (EMD Biosciences, San Diego, CA), recombinant human IL-4 (Peprotech, Rockhill, NJ), or recombinant human IL-13 (Peprotech) was also added to the cells. Before use, SAP was buffer-exchanged as described previously (Shao et al., 2008). To ligate surface receptors, PBMC were cultured in SFM with 2 μg/ml anti-human CD44 (clone G44-26) (BD Biosciences, San Jose, CA), anti-human CD44 (clone 515, an antibody that prevents HA binding to CD44) (Kansas et al., 1989; Siegelman et al., 1999) (BD Biosciences), anti-human CD43 (BD Biosciences), or mouse IgG1 isotype control (BD Biosciences). Dilutions of hyaluronic acid (or an equivalent volume of water as a control) were made in SFM. Cells were cultured in duplicate wells of a 96 well tissue culture plate (BD Biosciences). On day 5, fields of PBMC were photographed using a phase-contrast microscope with a 20 x objective and viable cells counted as described previously (Maharjan et al.,

2010). Cells were then fixed, stained, and the number of fibrocytes was counted, as described previously (Maharjan et al., 2010; Pilling et al., 2003; Pilling et al., 2006; Pilling et al., 2009; Shao et al., 2008). Fibrocytes were identified as adherent spindle-shaped cells with an oval nucleus as described previously (Maharjan et al., 2010; Pilling et al., 2003; Pilling et al., 2006; Pilling et al., 2009).

3.2.2 Treating hyaluronic acid with hyaluronidase

3 mg/ml of HMWHA or 3 mg/ml of LMWHA in PBS were treated with 10 U/ml of hyaluronidase from *Streptomyces hyalurolyticus* (Sigma-Aldrich) for 1 or 4 hours at 37^{o}C, and the digestion was stopped by boiling the solution for 5 minutes at 100^{o}C. Electrophoresis of HA on agarose gels was done following (Lee and Cowman, 1994) with the exception that 10 µl of sample was mixed with 5 µl of 6 x DNA loading buffer (Sharp et al., 1973), and 40 µl of 1 Kb DNA ladder (G5711, Promega, Madison, WI) was mixed with 10 µl of 6 x DNA loading buffer (Sharp et al., 1973). 15 µl of samples or DNA ladder were loaded on the gel, and this was run at 40 V for 8-10 hours (Lee and Cowman, 1994). The gels were stained with 0.005% Stains-All (Sigma-Aldrich) in 50% ethanol/water (Lee and Cowman, 1994). Dilutions of the digested hyaluronic acids (or PBS as a control) were made in SFM.

3.2.3 Detecting DNA, RNA, protein, or endotoxin contamination in HMWHA, LMWHA, or digested HMWHA

1 µg/ml 1 Kb DNA ladder, 10 µg/ml, 1 µg/ml, or 0.1 µg/ml of HMWHA, LMWHA, or digested HMWHA were electrophoresed on 1% agarose gels containing 2 µg/ml ethidium bromide for 2 hours at 40 V. The gels were visualized by UV and photographed. We also examined 10 ng/ml, 100 ng/ml, or 1000 ng/ml of HMWHA, or digested HMWHA at 260/280 nm on a Synergy MX (Biotek, Winooski, VT) microplate reader using a Take3 UV plate to examine the presence of DNA, RNA, or protein contamination, using BSA as a protein control. The detection limit of the Synergy MX is 2 ng/µl dsDNA, 2 ng/µl RNA, or 6 ng/µl protein. We tested for the presence of endotoxin in digested HMWHA using THP-1 blue cells (Invivogen, San Diego, CA) and QuantiBlue (Invivogen) following the manufacturer's instructions.

3.2.4 Preparation of monocytes

CD16-negative monocytes were purified from 5 x 10^7 PBMC using an EasySep Monocyte Depletion Kit (StemCell Technology, Vancouver, Canada) following the manufacturer's instructions (Maharjan et al., 2010). To determine the purity of the monocytes, cells were analyzed by flow cytometry (FACScan, BD Biosciences, or Accuri C6 flow cytometer, Ann Arbor, MI), as described previously (Maharjan et al., 2010; Pilling et al., 2009 ; Pilling et al., 2009; Shao et

al., 2008). A sample of each monocyte preparation was stained with 5 µg/ml primary antibodies against CD3, CD14, CD16, CD19, and CD45, and then incubated with FITC-conjugated F(ab′)2 goat anti-mouse IgG antibodies (cross-adsorbed against human Ig, Southern Biotechnology, Birmingham, AL, USA) as described previously (Maharjan et al., 2010; Shao et al., 2008). Only negatively selected cells in excess of 98% pure were used, as determined by the positive expression of CD14 and CD45. Less than 1% of the cells showed staining for the T cell marker CD3, the NK cell marker CD16, or the B cell marker CD19. 100 µl of purified monocytes at 5 x 10^5 cells/ml in SFM was mixed with 100 µl of 600 µg/ml HMWHA in SFM, 600 µg/ml LMWHA in SFM, or a water dilution control. On day 5, the cells were fixed, stained, and the number of fibrocytes was counted.

3.2.5 Immunocytochemistry for fibrocytes

PBMC were cultured on eight-well glass slides (Lab-Tek, Nalge Nunc International, Naperville, IL) in the presence or absence of 300 µg/ml HMWHA or SFM for 5 days, as described previously (Pilling et al., 2009). Slides were then gently tilted to reduce dislodging cells and the media was then removed from the corner of the well. Immunocytochemistry was performed as described previously (Pilling et al., 2009) with antibodies against CD13 (BioLegend, San Diego, CA), CD14 (BioLegend), CD34 (BD Biosciences), CD45RO (BioLegend), CXCR4

(R&D Systems, Minneapolis, MN), and collagen I (Rockland Immunochemicals, Gilbertsville, PA).

3.2.6 Immunofluorescence and staining for hyaluronic acid

PBMC were cultured on eight-well glass slides (Lab-Tek, Nalge Nunc International, Naperville, IL) for 5 days, as described previously (Pilling et al., 2009 ; Shao et al., 2008). Slides were then gently tilted to reduce dislodging cells and the media was then removed from the corner of the well. 400 µl of TBS was then gently added to the wells and then gently pipetted out from the corner of the well. The cells were then fixed with 200 µl of 2% paraformaldehyde (PFA) in PBS for 15 minutes at room temperature. After the PFA was removed, 400 µl of ice-cold methanol was added to the wells for 1 hour at 4^{o}C to permeabilize the cells. After gently removing the methanol, 400 µl of TBS was added to the wells for 10 minutes at room temperature and then gently pipetted out from the corner of the well. This was repeated twice. 400 µl of TBS containing 5% BSA (TBS-5% BSA) was then added to the wells at room temperature for 60 minutes to reduce nonspecific binding. These PBMC slides were then incubated with 5 µg/ml mouse anti-human-CD44 (G44-26), rabbit anti-human-RHAMM (clone EPR4055 Epitomics, Burlingame, CA), or mouse IgG1, isotype control, at room temperature for 60 minutes. Wells were washed with 400 µl of TBS, and then incubated with 2.5 µg/ml FITC-conjugated F(ab′)2 goat anti-mouse or goat anti-rabbit IgG antibodies (Southern Biotechnology, Birmingham, AL) at room

temperature for 30 minutes. After washing the slides with TBS, the slides were mounted with Vectashield mounting media containing DAPI (Vector Laboratories, Burlingame, CA). Images of the cells were captured on an Axioplan2 microscope (Zeiss) with a CoolSNAP HQ digital camera (Photometrics, Tucson, AZ) and Metamorph software (Molecular Devices, Dowingtown, PA).

To stain for hyaluronic acid, purified human monocytes were cultured in the presence or absence of 300 µg/ml HMWHA or LMWHA in eight-well glass slides as above for 30 minutes. The media was gently pipetted out and cells were fixed, permeabilized, and non-specific binding reduced as above. The monocytes were then incubated with biotinylated hyaluronic acid binding protein (bio-HABP) (Northstar Associates, East Falmouth, MA) diluted 1:500 in TBS-5% BSA at room temperature for 60 minutes. Wells were washed with 400 µl of TBS, and then incubated with 1.0 µg/ml streptavidin-FITC (BD Biosciences) at room temperature for 30 minutes. After washing the slides with TBS, the slides were mounted as above.

3.2.7 Statistics

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Statistical significance was defined as $p < 0.05$ as determined by the statistical methods indicated in the figure legends.

3.3 Results

3.3.1 HMWHA potentiates fibrocyte differentiation whereas LMWHA inhibits fibrocyte differentiation

Hyaluronic acid is a negatively charged glycosaminoglycan that is abundantly present in extracellular matrix (Girish and Kemparaju, 2007; Kuo, 2005; Laurent and Fraser, 1992; Scheibner et al., 2006; Stern et al., 2006). To investigate the role of hyaluronic acid on fibrocyte differentiation, PBMC were cultured in the presence of two different sizes of hyaluronic acid. According to the manufacturer, HMWHA has a range of molecular masses with a peak at 2 x 10^6 Da and LMWHA has a range of molecular masses with a peak at 7.5 x 10^5 Da; as described below, these sizes were verified by gel electrophoresis. In our serum-free culture media, when PBMC were incubated with HMWHA or LMWHA, we found that 300 μg/ml HMWHA potentiated fibrocyte differentiation, whereas 300 μg/ml LMWHA inhibited fibrocyte differentiation (Figure 3.3.1). These data suggest that HMWHA and LMWHA have opposite effects on fibrocyte differentiation. All batches of HMWHA and LMWHA were tested for protein, RNA, DNA, and these were found to be below the level of detection (data not shown). PBMC treated with 300 μg/ml, 100 μg/ml, 0.03 μg/ml, or 0.01 μg/ml HMWHA or LMWHA had no significant difference in the number of viable cells at 5 days compared to untreated cells (Figure 3.3.2). These data suggest that HMWHA and LMWHA have opposite effects on fibrocyte differentiation without affecting total cell viability.

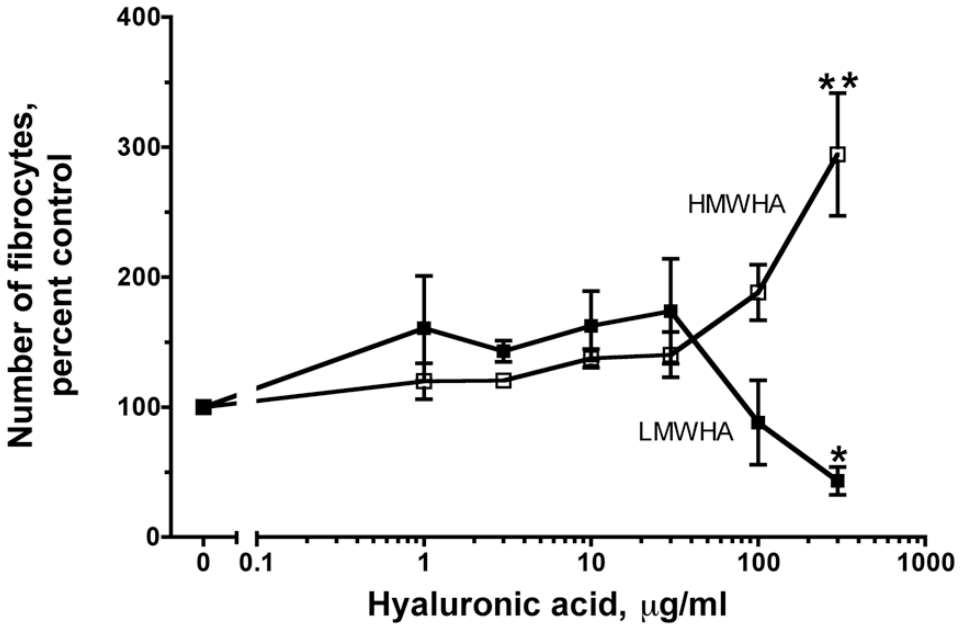


Figure 3.3.1 High molecular weight hyaluronic acid (HMWHA) promotes fibrocyte differentiation, while low molecular weight hyaluronic acid (LMWHA) inhibits fibrocyte differentiation. Human PBMC were cultured in the indicated concentrations of HMWHA and LMWHA. After 5 days, the cells were air dried, fixed, stained, and the number of fibrocytes was counted. Fibrocytes were identified as adherent spindle-shaped cells with an oval nucleus. For each donor, values were calculated as the percent of the no-HA control. The results are mean ± SEM (n = 6 separate experiments for HMWHA and n = 5 separate experiments for LMWHA). The number of fibrocytes per 2.5 x 10^5 PBMC for the no-HA controls from the 6 donors was 808, 400, 296, 450, 850, and 1200. * indicates $p < 0.05$ and ** $p < 0.01$, compared to control as determined by t-test.

Using the non-parametric Mann Whitney two-tailed t-test, 300 μg/ml and 100 μg/ml HMWHA significantly increases the number of fibrocytes when compared to control with $p < 0.001$, and 300 μg/ml LMWHA significantly decreases the number of fibrocytes when compared to control with $p < 0.01$.

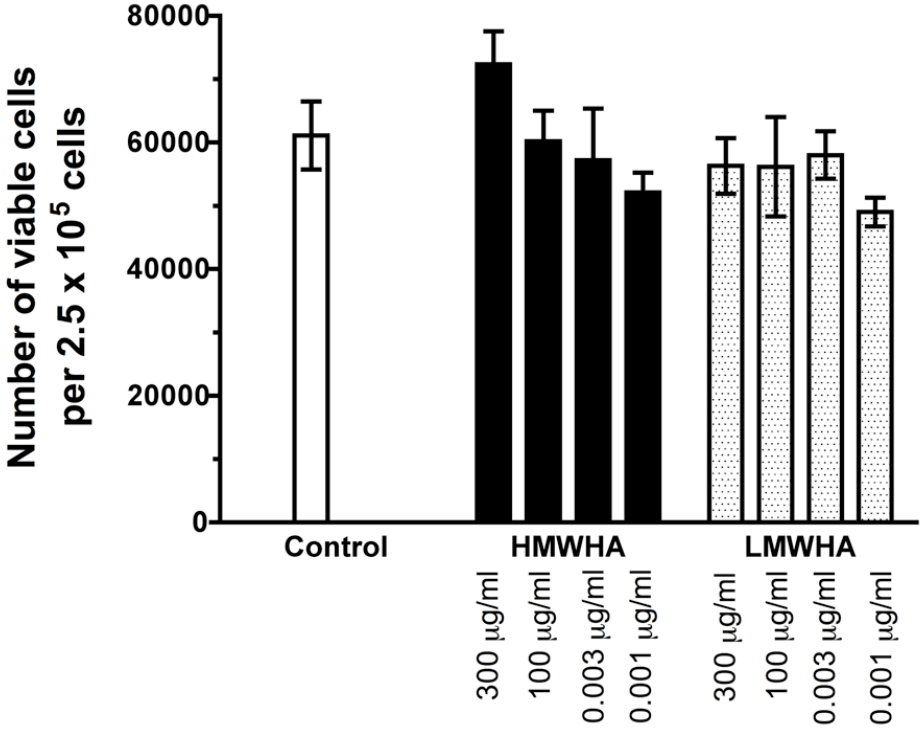


Figure 3.3.2. HMWHA and LMWHA do not affect cell viability. Human PBMC were cultured in the absence (SFM) or presence of the indicated concentrations of HMWHA or LMWHA for 5 days. After 5 days, the number of cells was counted. The results are mean ± SEM of viable cells per 0.15 mm^2 (n = 4 separate experiments). There was no statistical significance as analyzed by ANOVA or t-test.

To confirm that the long spindle-shaped cells are fibrocytes, I stained day 5 PBMC incubated with 300 μg/ml HMWHA or SFM for fibrocyte markers such as CD13, CD34, CD45RO, CXCR4, and collagen I. When PBMC were cultured in the presence of 300 μg/ml HMWHA or SFM for 5 days, the long spindle-shaped cells stained positively for CD13, CD34, CD45RO, CXCR4, and collagen I (data not shown). I also stained the day 5 PBMC cultured in 300 μg/ml HMWHA or SFM for the monocyte marker CD14 and the macrophage marker PM2K. Fibrocytes from both conditions did not stain for CD14 or PM2K (data not shown). There were few non-fibrocyte cells that stained positively for CD14 and PM2K (data not shown). Together, these observations indicate that the long spindle-shaped cells are fibrocytes.

3.3.2 Hyaluronidase treated HA inhibits fibrocyte differentiation

Since HMWHA potentiated fibrocyte differentiation and LMWHA inhibited fibrocyte differentiation, it is possible that HMWHA contains a contaminant that potentiates fibrocyte differentiation, or LMWHA contains a contaminant that inhibits fibrocyte differentiation. To confirm that hyaluronic acid of different sizes have different effects, we digested HMWHA into smaller fragments with hyaluronidase to determine if digested HMWHA inhibits fibrocyte differentiation. HMWHA had a range of molecular mass of approximately 6.3×10^6 Da - 2×10^6 Da (Figure 3.3.3A). The size range of the HMWHA digested with hyaluronidase was equivalent to the size of LMWHA (a mass range of approximately 6×10^5 Da

- 8 x 10^5 Da) as analyzed by gel electrophoresis, while hyaluronidase treatment of LMWHA slightly decreased the upper end of the size range of the LMWHA (Figure 3.3.3A). Hyaluronidase may have digested the HMWHA and LMWHA into smaller sizes such as oligo hyaluronic acid. However, I was unable to detect these smaller sizes on the gel. Hyaluronidase-treated HMWHA and LMWHA inhibited fibrocyte differentiation (Figure 3.3.3B). Hyaluronidase-containing PBS had no effect on fibrocyte differentiation (data not shown). Since HMWHA were digested with bacterial hyaluronidase, it is possible that the digested HMWHA may contain endotoxins that are actually causing the inhibition of fibrocyte differentiation. Hyaluronidase digested HMWHA always had endotoxin below detectable levels (data not shown). Treatment of PBMC with HMWHA and LMWHA digested with hyaluronidase also did not affect cell viability at 5 days (data not shown). Together, this suggests that the potentiation of fibrocyte differentiation by HMWHA and the inhibition of differentiation by LMWHA are not due to a non-HA contaminant.

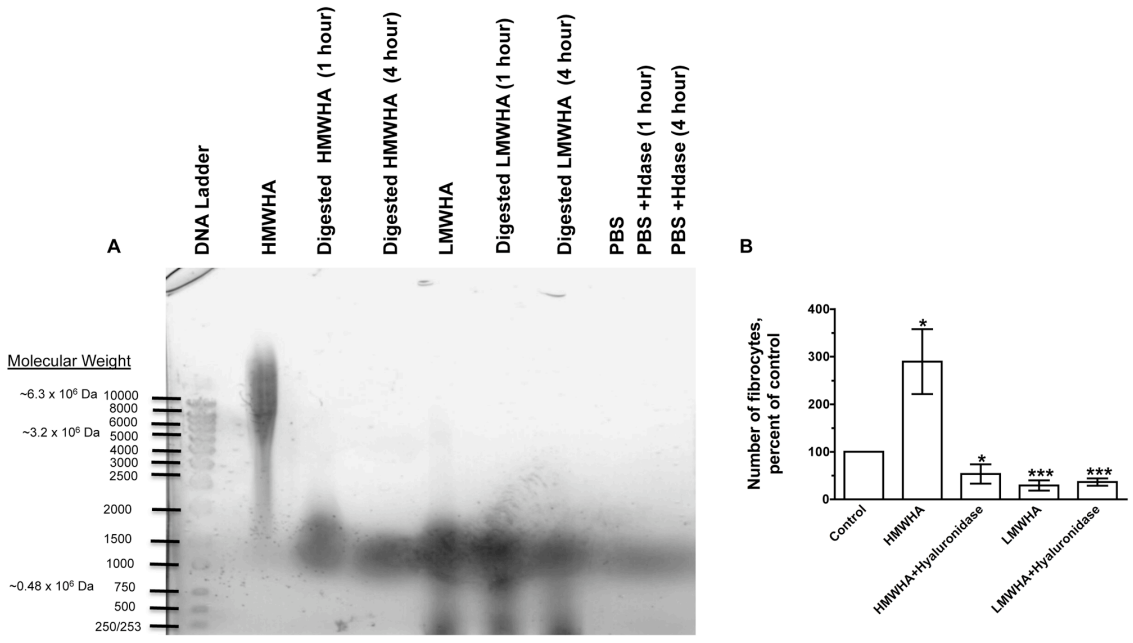


Figure 3.3.3. HMWHA and LMWHA treated with hyaluronidase inhibit fibrocyte differentiation. (A) HMWHA and LMWHA were treated with 10 U/ml of hyaluronidase for 1 and 4 hours at 37°C. Samples were analyzed by agarose gel electrophoresis. M, 1 Kb ladder; Lane 1, HMWHA; Lane 2, HMWHA incubated with hyaluronidase for 1 hour; Lane 3, HMWHA incubated with hyaluronidase for 4 hours; Lane 4, LMWHA; Lane 5, LMWHA incubated with hyaluronidase for 1 hour; Lane 6, LMWHA incubated with hyaluronidase for 4 hours; Lane 7, PBS; Lane 8, PBS incubated with hyaluronidase for 1 hour; and Lane 9, PBS incubated with hyaluronidase for 4 hours. (B) Human PBMC were cultured in 300 µg/ml of 4 hours-hyaluronidase-treated or untreated HMWHA and LMWHA. After 5 days, the cells were air dried, fixed, stained, and the number of fibrocytes was counted. The results are mean ± SEM of SFM percent control (n = 6 separate

experiments). The number of fibrocytes in SFM controls from the 6 donors was 883, 1029, 808, 288, 400, and 288. * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as determined by t-test. Using the non-parametric Mann Whitney two-tailed t-test, 300 μg/ml HMWHA significantly increases the number of fibrocytes when compared to control with $p < 0.001$, but 300 μg/ml hyaluronidase incubated HMWHA is not significantly different from control. Also, 300 μg/ml LMWHA significantly decreases the number of fibrocytes when compared to control with $p < 0.001$, and 300 μg/ml hyaluronidase incubated LMWHA also significantly decreases the number of fibrocytes when compared to control with $p < 0.001$. Using the non-parametric Mann Whitney one-tailed t-test, 300 μg/ml hyaluronidase incubated HMWHA significantly decreases the number of fibrocytes when compared to control with $p < 0.05$.

High and low molecular weight HA are present at sites of injury (Bollyky et al., 2007; Jiang et al., 2007; Powell and Horton, 2005). I therefore examined how combinations of 300 μg/ml HMWHA and 300 μg/ml LMWHA affect fibrocyte differentiation. The number of fibrocytes observed in PBMC treated with a combination of HMWHA and LMWHA was significantly lower compared to PBMC cultured with HMWHA alone, but not significantly different compared to PBMC cultured with LMWHA alone (Figure 3.3.4). In this experiment, I added 2.7 times more moles of LMWHA than HMWHA. It is possible that the increased amount of LMWHA that I added neutralized the effect of HMWHA on fibrocyte differentiation. When we analyze the data with Mann Whitney one-tailed t-tests, I observe that 300 μg/ml HMWHA or 300 μg/ml LMWHA are significantly different from the combination of 300 μg/ml HMWHA and LMWHA, which suggests that HMWHA and LMWHA both neutralize each other's effect on fibrocyte differentiation.

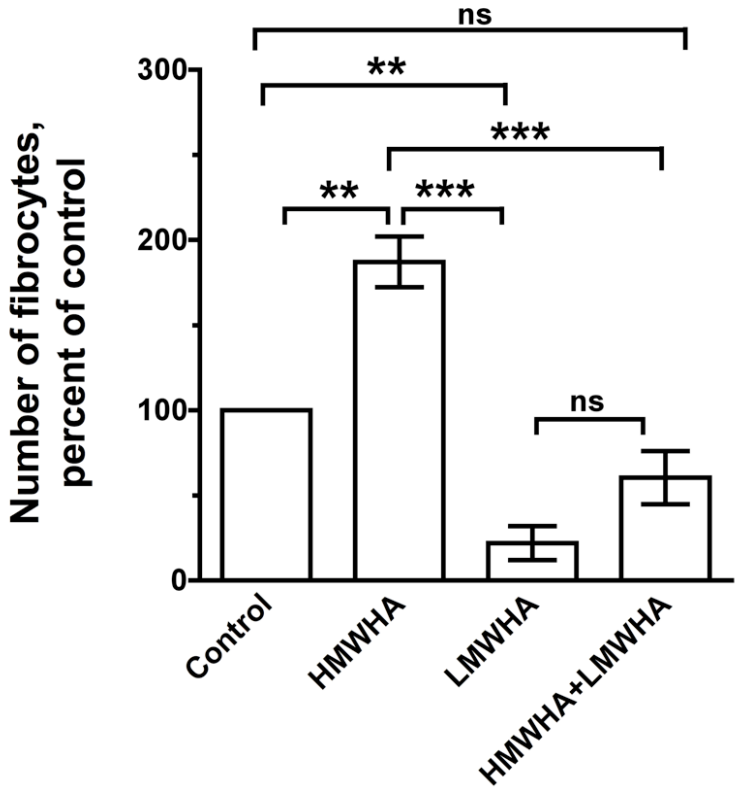


Figure 3.3.4. LMWHA inhibits HMWHA-induced fibrocyte differentiation. Human PBMC were cultured in SFM (control), SFM with 300 μg/ml HMWHA, SFM with 300 μg/ml LMWHA, or SFM with 300 μg/ml HMWHA and 300 μg/ml LMWHA. After 5 days, the cells were air dried, fixed, stained, and the number of fibrocytes was counted. The results are mean ± SEM of percent of control (n = 3 separate experiments). The number of fibrocytes per 2.5 x 10^5 PBMC in controls from the 3 donors was 1525, 713, and 1290. * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control as determined by ANOVA. Using the non-parametric Mann Whitney two-tailed t-test, there is no statistical significant difference among different experimental groups. Using the non-parametric Mann Whitney one-

tailed t-test, 300 μg/ml HMWHA significantly increases the number of fibrocytes when compared to control with $p < 0.05$, 300 μg/ml LMWHA significantly decreases the number of fibrocytes when compared to control with $p < 0.05$, 300 μg/ml HMWHA and 300 μg/ml LMWHA significantly decreases the number of fibrocytes when compared to control with $p < 0.05$. Additionally, 300 μg/ml HMWHA significantly increases the number of fibrocytes when compared to 300 μg/ml LMWHA or the combination of 300 μg/ml HMWHA and LMWHA with $p < 0.05$. 300 μg/ml LMWHA significantly decreases the number of fibrocytes when compared to 300 μg/ml HMWHA or the combination of 300 μg/ml HMWHA and LMWHA with $p < 0.05$.

3.3.3 HMWHA and LMWHA directly affect the differentiation of monocytes to fibrocytes

We previously found that IL-12 and TLR2 agonists, when added to a population of PBMC, indirectly affect monocyte to fibrocyte differentiation (Maharjan et al., 2010; Shao et al., 2008), while factors such as SAP, IL-4, IL-13, or aggregated IgG directly affect monocyte to fibrocyte differentiation (Pilling et al., 2003; Pilling et al., 2006; Shao et al., 2008). Therefore, I examined whether HMWHA or LMWHA act directly on human monocytes. When 300 μg/ml HMWHA or LMWHA were added to purified monocytes, HMWHA potentiated fibrocyte differentiation while LMWHA inhibited fibrocyte differentiation (Figure 3.3.5). This suggests that HMWHA and LMWHA act directly on monocytes to either potentiate or inhibit fibrocyte differentiation.

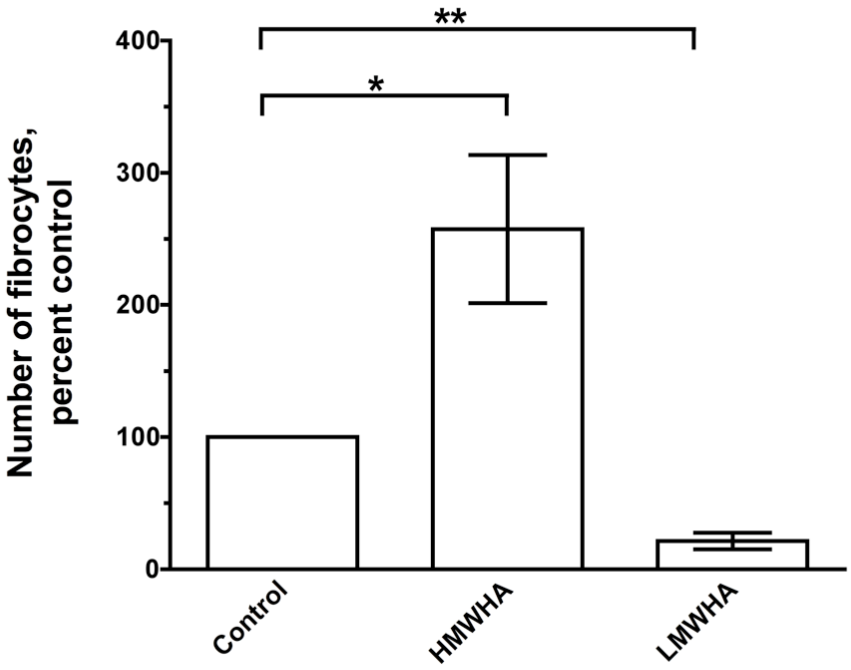


Figure 3.3.5. HMWHA potentiates but LMWHA inhibits the differentiation of purified monocytes to fibrocytes. Purified human monocytes were cultured in 300 μg/ml of either HMWHA or LMWHA for 5 days. After 5 days, the cells were air dried, fixed, stained, and the number of fibrocytes was counted. The results are mean ± SEM of percent no-HA control (n = 3 separate experiments). The number of fibrocytes per 2.5 x 10^5 monocytes in controls from the 3 donors was 4250, 2450, and 1600. * indicates $p < 0.05$ and ** indicates $p < 0.01$ compared to control as determined by t-test. Using the non-parametric Mann Whitney two-tailed t-test, there is no significant difference among the control, 300 μg/ml HMWHA, or 300 μg/ml LMWHA. Using the non-parametric Mann Whitney one-tailed t-test, 300 μg/ml HMWHA significantly increases the number of fibrocytes

when compared to control, 300 µg/ml LMHWA significantly decreases the number of fibrocytes when compared to control.

3.3.4 The pro-fibrocyte effect of HMWHA varies with different cell density

Since HMWHA and LMWHA directly affect the differentiation of monocytes to fibrocytes, it is possible that HMWHA potentiates the number of fibrocytes while LMWHA inhibits the number of fibrocytes because HMWHA can cause cell-cell interactions due to its large size. An alternative hypothesis is that HMWHA potentiates fibrocyte differentiation because it has more PBMC binding sites due to its length. To test these hypotheses, I incubated PBMCs with HMWHA at three different cell densities. At 0.125 x 10^6 cells/ml PBMC, HMWHA potentiated fibrocyte differentiation more than 0.5 x 10^6 cells/ml or 2.0 x 10^6 cells/ml (Figure 3.3.6). At 2.0 x 10^6 cells/ml, PBMCs clumped together and probably interfered with fibrocyte differentiation. HMWHA potentiated more fibrocytes in 0.125 x 10^6 cells/ml than in 0.5 x 10^6 cells/ml cultures, which suggests that the potentiation of fibrocyte differentiation by HMWHA is not due to cell-cell interactions due to the large size of HMWHA.

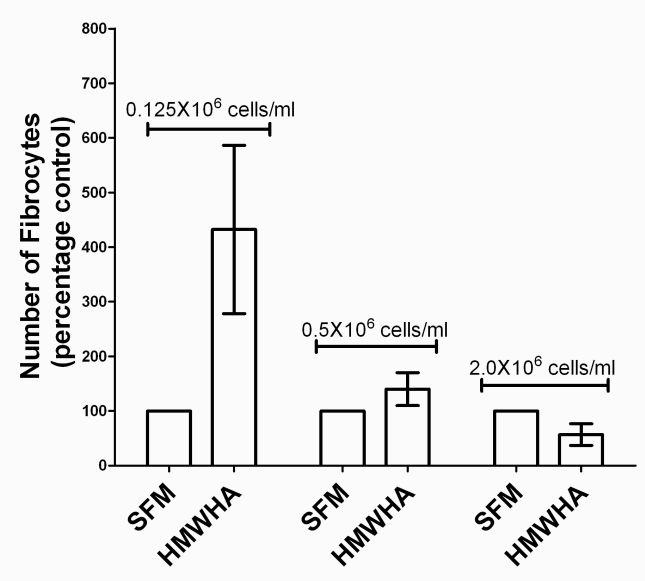


Figure 3.3.6: The pro-fibrocyte effect of HMWHA varies with different cell density. Human PBMC at different cell densities were cultured in serum-free medium for 5 days in the presence of 300 µg/ml HMWHA. The results are mean ± SEM of fibrocytes (n = 3 separate experiments).

3.3.5 Oligo hyaluronic acid has no effect on fibrocyte differentiation.

Since LMWHA and hyaluronidase-treated HMWHA decreased the number of fibrocytes, I investigated the possibility that very small hyaluronic acid polymers might inhibit fibrocyte differentiation. When PBMC were cultured with increasing concentrations of 6-mer oligo hyaluronic acid (1,230 Da), there was no difference in the number of fibrocytes compared to SFM (Figure 3.3.7). This indicates that although LMWHA inhibits fibrocyte differentiation, the effect requires a polymer larger than a 6-mer/1,230 Da hyaluronic acid. This also suggests that hyaluronidase digested HMWHA inhibited fibrocyte differentiation (Figure 3.3.3B) due to a size that is similar to LMWHA, and not because of the small fragment sizes that are similar to oligo hyaluronic acid.

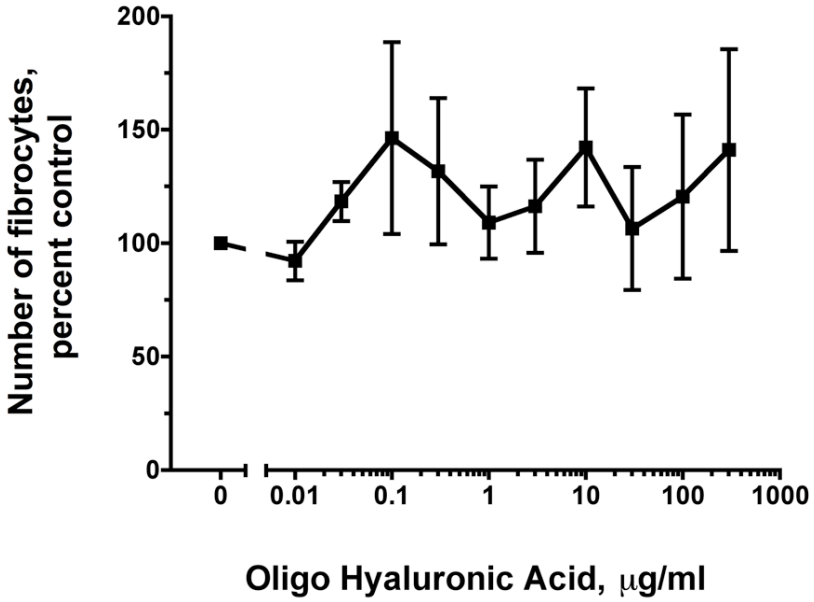


Figure 3.3.7: Oligo hyaluronic acid does not affect fibrocyte differentiation. Human PBMC were cultured in different concentrations of oligo hyaluronic acid for 5 days. After 5 days, the cells were air dried, fixed, stained, and the number of fibrocytes was counted. The results are mean ± SEM of percent control (n = 3 separate experiments). The number of fibrocytes 2.5 x 10^5 PBMC in controls from the 3 donors was 4150, 2560, and 575.

3.3.6 HMWHA and LMWHA have different effects on the expression of CD44

Hyaluronic acid binds to the cell-surface molecule CD44, and this interaction plays an important role in development, inflammation, tumor growth, and the recruitment and activation of many immune cells into injured tissues (Lesley et al., 1993). Therefore I tested the effect of HMWHA and LMWHA on the surface levels of CD44 on PBMC. After culturing PBMC in the presence or absence of 300 μg/ml of HMWHA or 300 μg/ml of LMWHA for 5 days, cells were stained for CD44. For PBMC cultured in SFM, both fibrocytes and non-fibrocyte cells expressed CD44 (Figure 3.3.8). In cultures containing HMWHA, fibrocytes and non-fibrocyte cells showed staining for CD44 similar to the control (Figure 3.3.8). In cultures containing LMWHA, both fibrocytes and non-fibrocyte cells had less staining for CD44 compared to the control (Figure 3.3.8). These results indicate that not only do HMWHA and LMWHA have opposite effects on fibrocyte differentiation, but they also have different effects on the levels of CD44 on fibrocytes and non-fibrocyte cells.

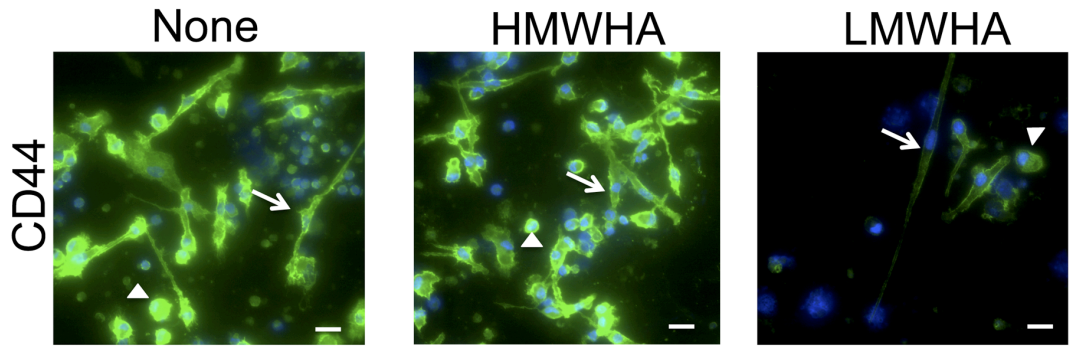


Figure 3.3.8. HMWHA and LMWHA have different effects on the expression of CD44. PBMC were incubated in SFM, SFM with 300 µg/ml HMWHA, or SFM with 300 µg/ml LMWHA for 5 days. The PBMC were then fixed and stained for CD44. In all panels, nuclei are stained blue with DAPI and bars are 20 µm. Arrows indicate fibrocytes and arrowheads indicate non-fibrocyte cells. The figures represent one of 3 independent experiments.

3.3.7 Anti-human CD44 antibodies potentiate fibrocyte differentiation

To determine whether the hyaluronic acid binding receptor CD44 could directly affect fibrocyte differentiation, I cultured PBMC with two different anti-human CD44 antibodies, anti-human CD43, or mouse IgG1 isotype control for 5 days. I used CD43 as a second hematopoietic marker as it plays an important role in adhesion, cell-signaling, and cytoskeleton interactions, but does not bind HA (Nong et al., 1989). One of the anti-human CD44 antibodies (clone 515) is a blocking antibody that binds at or near the same site as HA on CD44, preventing HA from binding to CD44 (Kansas et al., 1989; Siegelman et al., 1999). The second CD44 mAb (clone G44-26) does not prevent HA binding to CD44. When added to PBMC, both CD44 antibodies significantly increased the number of fibrocytes compared to PBMC cultured in either SFM or mouse IgG1 antibodies (Figure 3.3.9). Anti-human CD43 and mouse IgG1 isotype control antibodies had no statistically significant effect on fibrocyte differentiation.

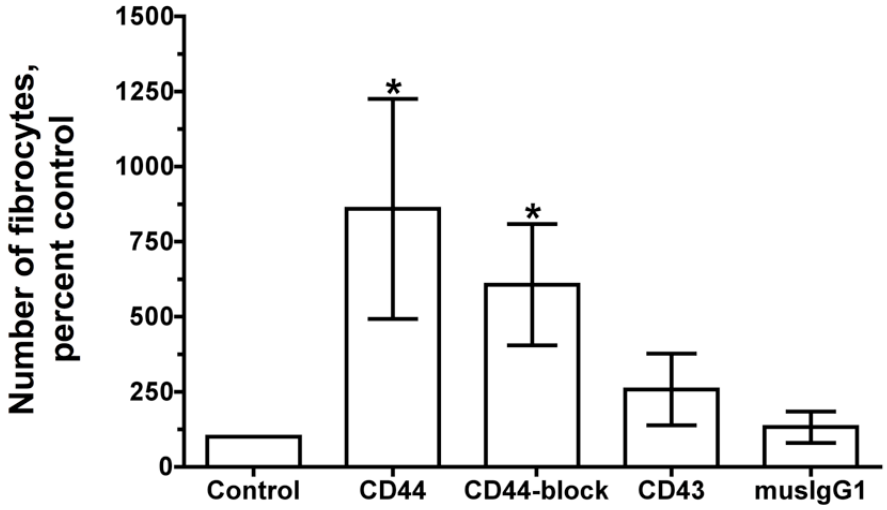


Figure 3.3.9. Anti-CD44 antibodies potentiate the differentiation of monocytes to fibrocytes. Human PBMC were cultured in 2 μg/ml anti-human anti-CD44, anti-CD43, or mouse IgG1 isotype control. After 5 days, the cells were air dried, fixed, stained, and the number of fibrocytes was counted. The results are mean ± SEM of percent control (n = 6 separate experiments). The number of fibrocytes per 2.5 x 10^5 PBMC in controls was 350, 50, 350, 250, 450, and 50. * indicates $p < 0.05$ compared to control, as determined by ANOVA with Dunnett's post-test). Using the Mann Whitney two-tailed t-test, 2 μg/ml anti-human anti-CD44 antibodies significantly increases the number of fibrocytes when compared to control with $p < 0.001$.

HA can also bind to other surface receptors such as RHAMM (Hardwick et al., 1992; Nedvetzki et al., 2004). I examined if HMWHA or LMWHA affect the expression of RHAMM. After culturing PBMC in the presence or absence of 300 μg/ml of HMWHA or 300 μg/ml of LMWHA for 5 days, cells were stained for RHAMM. As described previously in activated monocytes (Weiss et al., 1998) or $CD34^+$ cells (Greiner et al., 2002), I did not observe any staining for RHAMM in fibrocytes or non-fibrocyte cells in the presence or absence of HMWHA or LMWHA (data not shown).

3.3.8 Purified monocytes internalize HMWHA and LMWHA

As HMWHA and LMWHA directly affect the differentiation of monocytes to fibrocytes, I examined if the internalization of HMWHA and LMWHA was different in purified monocytes. Purified monocytes were incubated with or without 300 μg/ml of HMWHA or 300 μg/ml of LMWHA, and after 30 minutes the cells were fixed. The fixed cells were then stained for hyaluronic acid. I detected a low intensity fluorescence signal on untreated monocytes, which could have been due to autofluorescence, non-specific binding of the biotinylated hyaluronic acid binding protein (bio-HABP), or the presence of HA bound to the cells ex vivo, as these were freshly prepared cells, and HA is present in plasma at 0.01-0.03 μg/ml (Engstrom-Laurent et al., 1985). I observed HA staining inside HMWHA and LMWHA treated monocytes (Figure 3.3.10). Monocytes treated with HMWHA or LMWHA for 60 minutes also had HA staining inside the cells (data

not shown). This suggests that the opposite effect of HMWHA and LMWHA on fibrocyte differentiation is unlikely to be due to differential internalization of high and low molecular weight hyaluronic acids by purified monocytes. Although ten-day cultured fibrocytes synthesize hyaluronic acid (Bianchetti et al., 2011), our inability to detect hyaluronic acid on monocytes cultured for 60 minutes suggest that hyaluronic acid production in fibrocytes is developmentally regulated.

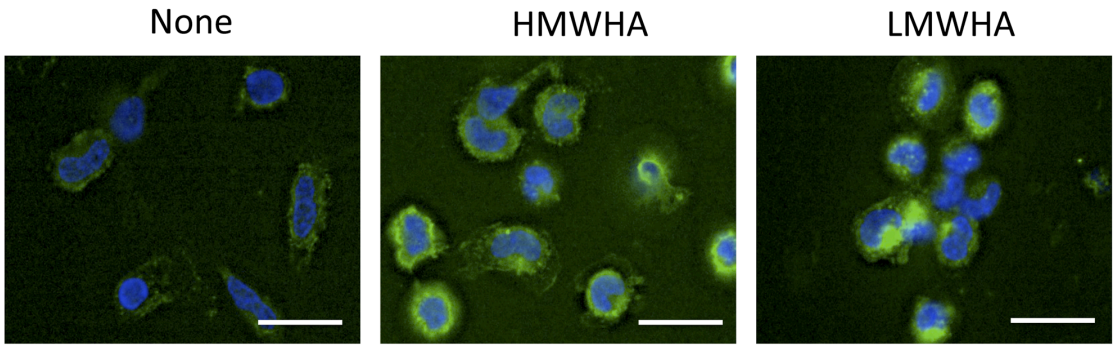
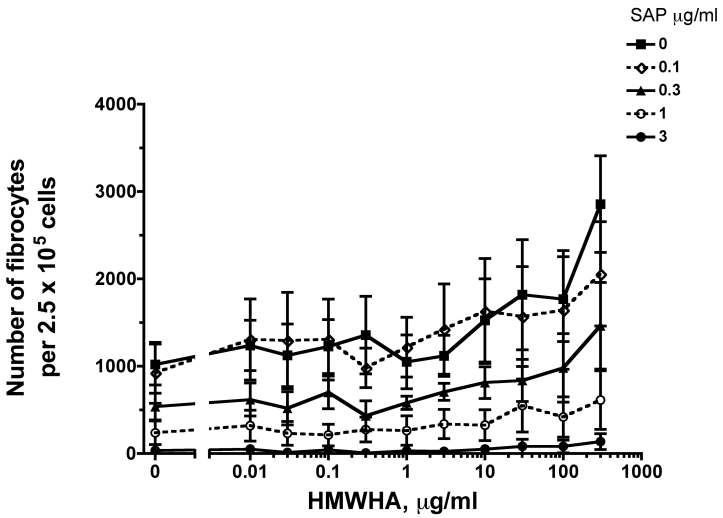


Figure 3.3.10. At 30 minutes, both LMWHA and HMWHA are internalized by monocytes. Purified human monocytes were incubated in SFM, SFM with 300 µg/ml LMWHA, or 300 µg/ml of HMWHA for 30 minutes. The PBMC were stained with biotinylated hyaluronic acid binding protein (HABP) and this was detected with streptavidin-FITC. In all panels, nuclei are stained blue with DAPI and bars are 20 µm. The figures represent one of the 3 independent experiments.

3.3.9 SAP inhibits HMWHA-induced fibrocyte differentiation and potentiates LMWHA-induced fibrocyte inhibition

A variety of pro-fibrocyte and anti-fibrocyte factors are present during tissue injury (Jiang et al., 2007; Niedermeier et al., 2009; Shao et al., 2008; Trujillo et al., 2010; Wang et al., 2007a). I investigated the effect of HMWHA and LMWHA on fibrocyte differentiation in the presence of SAP, a potent inhibitor of fibrocyte differentiation (Murray et al., 2011; Murray et al., 2010; Naik-Mathuria et al., 2008; Pilling et al., 2003; Pilling et al., 2009 ; Pilling et al., 2007; Pilling et al., 2009; Shao et al., 2008). PBMC were cultured in different concentrations of HMWHA or LMWHA along with different concentrations of SAP. SAP counteracted HMWHA-induced fibrocyte differentiation, and potentiated LMWHA-induced fibrocyte inhibition (Figure 4.3.11). This suggests that the fibrocyte-inhibiting activity of SAP has a dominant effect over the fibrocyte-promoting activity of HMWHA, and that LMWHA potentiates the ability of SAP to inhibit fibrocyte differentiation.

A



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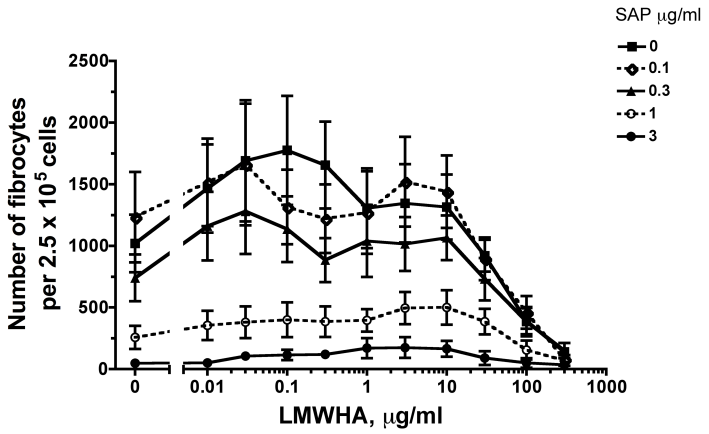


Figure 3.3.11. SAP inhibits HMWHA-induced fibrocyte differentiation and potentiates LMWHA-induced fibrocyte inhibition. (A) Human PBMC were cultured in different concentrations of HMWHA with 0, 0.1, 0.3, 1, and 3 μg/ml of SAP. After 5 days, the cells were air dried, fixed, stained, and the number of fibrocytes was counted. The results are mean ± SEM of fibrocytes per 2.5 x 10^5 PBMC (n = 5 separate experiments, except n = 11 separate experiments for

HMWHA with no SAP). With no SAP, the difference between no treatment and 300 μg/ml HMWHA is significant with $p < 0.01$ (t-test). With 300 μg/ml HMWHA, adding 1 or 3 μg/ml SAP significantly decreases the number of fibrocytes with $p < 0.05$ (ANOVA). Using the non-parametric Mann Whitney two-tailed t-test, with no SAP, the difference between no treatment and 300 μg/ml HMWHA is significant with $p < 0.05$. With 300 μg/ml HMWHA, adding 1 or 3 μg/ml SAP significantly decreases the number of fibrocytes with $p < 0.05$. (B) Human PBMC were cultured in different concentrations of LMWHA with 0, 0.1, 0.3, 1, and 3 μg/ml of SAP for 5 days. After 5 days, the cells were air dried, fixed, stained, and the number of fibrocytes was counted. The results are mean ± SEM of fibrocytes per 2.5×10^5 PBMC (n = 5 separate experiments, except n = 11 separate experiments for LMWHA with no SAP). With no added SAP, the difference between no treatment and 100 μg/ml or 300 μg/ml LMWHA is significant with $p < 0.001$ (t-test). Using the non-parametric Mann Whitney two-tailed t-test, with no added SAP, the difference between no treatment and 100 μg/ml or 300 μg/ml LMWHA is significant with $p < 0.05$.

3.3.10 LMWHA inhibits IL-4 or IL-13-induced fibrocyte differentiation

IL-4 and IL-13 are cytokines that potentiate fibrocyte differentiation (Pilling et al., 2003; Pilling et al., 2009 ; Shao et al., 2008). To determine if there is a regulatory hierarchy, I examined the effect of LMWHA on fibrocyte differentiation in the presence of IL-4 or IL-13. IL-4 and IL-13 promoted fibrocyte differentiation, and LMWHA inhibited IL-4- and IL-13-induced fibrocyte differentiation (Figure 3.3.12). This suggests that the fibrocyte inhibiting activity of LMWHA has a dominant effect over the fibrocyte promoting activities of IL-4 and IL-13.

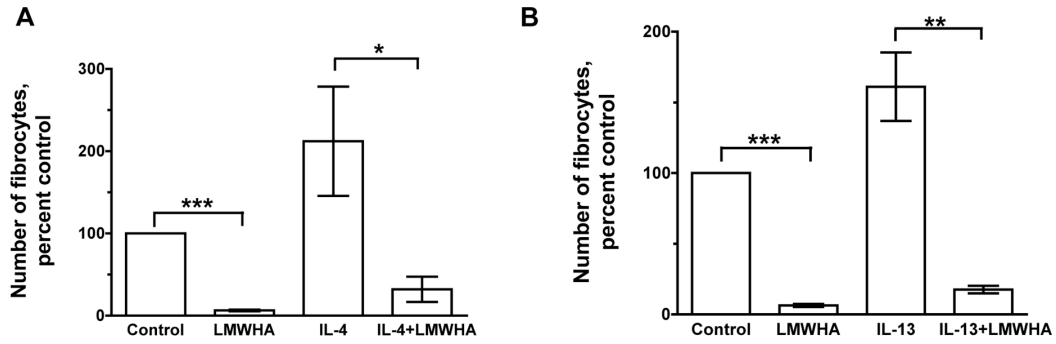


Figure 3.3.12. LMWHA inhibits IL-4 or IL-13-induced fibrocyte differentiation. Human PBMC were cultured in (A) SFM, SFM with 1 ng/ml IL-4, SFM with 300 µg/ml LMWHA, or SFM with 1 ng/ml IL-4 and 300 µg/ml LMWHA, or (B) SFM, SFM with 1 ng/ml IL-13, or SFM with 1 ng/ml IL-13 and 300 µg/ml LMWHA. After 5 days, the cells were air dried, fixed, stained, and the number of fibrocytes was counted. The results are mean ± SEM of percent control (n = 3 separate experiments). The number of fibrocytes per 2.5 x 10^5 PBMC in controls from the 3 donors was 900, 1520, and 431. * indicates $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to control as determined by t-test. Using the non-parametric Mann Whitney two-tailed t-test, there is no significant difference among control, 300 µg/ml LMWHA, 1 ng/ml IL-4, 1 ng/ml IL-13, the combination of 300 µg/ml LMWHA and 1 ng/ml IL-4, or the combination of 300 µg/ml LMWHA and 1 ng/ml IL-13. Using the non-parametric Mann Whitney one-tailed t-test, 300 µg/ml LMWHA significantly decreases the number of fibrocytes when compared to control with $p < 0.05$. Also, the combination of 300 µg/ml LMWHA and 1 ng/ml

IL-4 significantly decreases the number of fibrocytes when compared to 1 ng/ml IL-4 with $p < 0.05$. The combination of 300 μg/ml LMWHA and 1 ng/ml IL-13 also significantly decreases the number of fibrocytes when compared to 1 ng/ml IL-13 with $p < 0.05$.

3.4 Discussion

I found that 300 μg/ml HMWHA potentiates fibrocyte differentiation and 300 μg/ml LMWHA inhibits fibrocyte differentiation. These HA concentrations are similar to what has been observed in tissues (Kuo, 2005), and the HA concentrations used in other studies (Forrester and Balazs, 1980; Grishko et al., 2009; McKee et al., 1996; Scheibner et al., 2006; Teder et al., 2002). The effect of these two different sizes of hyaluronic acid on fibrocyte differentiation is a direct effect on monocytes. HMWHA and LMWHA also have different effects on the levels of the adhesion receptor CD44. The opposite effect of HMWHA and LMWHA on fibrocyte differentiation does not appear to be due to the difference in the internalization of HMWHA and LMWHA by monocytes. Anti-human CD44 antibodies potentiate fibrocyte differentiation, suggesting that CD44 may at least in part mediate the effect of hyaluronic acid on fibrocyte differentiation. SAP inhibits HMWHA-induced fibrocyte differentiation and potentiates LMWHA-induced fibrocyte differentiation, while LMWHA inhibits IL-4- or IL-13-potentiated fibrocyte differentiation. These results suggest a dominance hierarchy of SAP > LMWHA ≥ HMWHA > IL-4 or IL-13.

HMWHA and LMHWA bind to CD44, TLR2, TLR4, LYVE, and RHAMM (CD168) receptors to accomplish their biological effects (Powell and Horton, 2005). I have previously shown in chapter 2 that TLR2 and TLR4 agonists do not have a direct effect on monocyte to fibrocyte differentiation (Maharjan et al.,

2010). Therefore, it is unlikely that HMWHA and LMWHA regulate fibrocyte differentiation via TLR2 or TLR4. We and others have also failed to detect RHAMM expression on fibrocytes or human monocytes cultured ex vivo, although macrophage cell lines express RHAMM (Greiner et al., 2002; Weiss et al., 1998). Finally LYVE is a HA receptor expressed predominately by lymphatic endothelium (Johnson et al., 2007), and we have not detected LYVE on monocytes, macrophages, or fibrocytes (data not shown) (Pilling et al., 2009). As antibodies against CD44 potentiated fibrocyte differentiation, CD44 appears to be the dominant receptor for HA induced regulation of fibrocyte differentiation.

Several studies have described differential effects of HMWHA and LMWHA on different types of cells such as macrophages, dendritic cells, osteoclasts, and T cells (Bollyky et al., 2007; Chang et al., 2007; McKee et al., 1996; Nakamura et al., 2004; Scheibner et al., 2006; Termeer et al., 2002; Termeer et al., 2000). For example, in murine and human macrophages, HMWHA (6×10^6 Da) did not affect the secretion of pro-inflammatory chemokines or cytokines (McKee et al., 1996), while LMWHA ($< 5 \times 10^5$ Da) stimulated these cells to produce inflammatory chemokines such as macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-1β (MIP-1β), and monocyte chemoattractant protein-1 (MCP-1) (Hodge-Dufour et al., 1997; Kuang et al., 2007; McKee et al., 1996; Termeer et al., 2002). In cultured macrophages, HMWHA ($4.6 \times 10^5 – 2.8 \times 10^6$ Da) inhibited phagocytosis, while LMWHA ($9.0 \times$

10^4 Da) enhanced phagocytosis (Forrester and Balazs, 1980). The inhibition of macrophage phagocytosis was proportional to the molecular weight of the HA. These results suggest that LMWHA (< 5 x 10^5 Da) actively stimulate many types of cells at sites of injury to facilitate the clearance of debris and infectious agents, while HMWHA (> 10^6 Da), which is the dominant HA in resolving wounds, acts as a signal for repair.

During tissue injury, cirrhosis, and fibrosis, the concentration of HA increases in serum and tissue fluids, and this HA appears to be mainly LMWHA (Jiang et al., 2007; Scheibner et al., 2006; Stern et al., 2006). The normal range of HA in serum is 0.01-0.1 μg/ml, while in patients with liver injury the serum concentration of HA reaches 0.1-0.3 μg/ml (Engstrom-Laurent et al., 1985). In murine models of lung injury, the concentration of HA increased by 50% and the size of the accumulated HA was approximately 5.4 x 10^5 Da (Teder et al., 2002). As the injury resolved, the concentration of HA returned to basal levels and the size reverted to 1.4 x 10^6 – 3.1 x 10^6 Da, (Bai et al., 2005; Teder et al., 2002) similar to the size of HA in the lungs of control mice. These results show that following an insult, there is an increase in the concentration of small hyaluronic acid fragments. As the injury resolves, the concentration of HA decreases and the size returns back to normal (> 10^6 Da). Combined with our results, this suggests that the presence of LMWHA at an injured site would inhibit fibrocyte differentiation and collagen deposition to allow macrophages to freely move

about the injured site to phagocytose debris and clear any infectious agents. As wound healing progresses and the ratio of HMWHA to LMWHA increases (Laurent and Fraser, 1992), the HMWHA will promote monocyte to fibrocyte differentiation, leading to tissue repair with the fibrocytes secreting ECM and also stimulating ECM production by fibroblasts (Hartlapp et al., 2001; Trujillo et al., 2010; Wang et al., 2007b). However, there is still no known mechanism to explain how a simple repeating disaccharide of varying length has opposing effects on a same type of cell.

Chapter 4: Serum Amyloid P (SAP) inhibits neutrophil adhesion and diminishes neutrophil accumulation in mice models of acute respiratory distress syndrome (ARDS)

4.1 Introduction

Minor infections or injuries to tissues such as the lungs cause the damaged cells to recruit immune cells, including neutrophils and monocytes, to the injury site. The neutrophils and monocytes then remove cell debris and bacteria from the injured site (Piantadosi and Schwartz, 2004). After the injured sites are clear, the neutrophils and monocytes leave and other cells repair the wound.

In blood vessels, neutrophils are generally quiescent and circulate in the vessel using an adhesion molecule called CD62L to roll along the vessel wall (Edwards, 1994; Witko-Sarsat et al., 2000). When neutrophils get signals from injured sites, the neutrophils push through the blood vessel wall and enter the injured sites. Neutrophils are able to push through the blood vessel using an adhesion molecule called CD11b (Witko-Sarsat et al., 2000). Neutrophils can be activated by different components of damaged cells or through cytokines such as IL-8, TNF-α, or GM-CSF (Cassatella, 1999; Montecucco et al., 2008; Nathan, 1987; Weisbart et al., 1985; Yong and Linch, 1992). IL-8 triggers neutrophils to migrate into the injured sites (Cassatella, 1999). TNF-α and GM-CSF increase

neutrophil adherence, release of reactive oxygen species, and phagocytosis (Montecucco et al., 2008; Nathan, 1987; Weisbart et al., 1985; Yong and Linch, 1992). When neutrophils get activated, they increase the number of cell surface CD11b molecules and decrease the number of cell surface CD62L molecules (Hughes et al., 2006; Strieter and Kunkel, 1994). Once neutrophils are at injured sites, they release reactive oxygen species and proteases, and then engulf bacteria and debris by phagocytosis (Hughes et al., 2006; Strieter et al., 1992).

In a normal wound repair resolution, activated neutrophils undergo programmed cell death, which prevents the release of reactive oxygen species from the neutrophils, thereby preventing any cell damage (Lee and Downey, 2001). These apoptotic neutrophils are usually cleared by phagocytic cells such as macrophages (Rossum et al., 2005).

Since activated neutrophils can cause so much damage to lung cells, we need to understand what factors affect neutrophil recruitment and activation. Factors such as bacteria and cell debris activate neutrophils and cause the release of reactive oxygen species. Neutrophils and monocytes have receptors called Fcγ receptors that can recognize bacteria and debris (Tsuboi et al., 2008). Fcγ receptors bind to aggregated IgGs, such as IgGs that are attached to bacteria. Fcγ receptors can also bind to proteins such as serum amyloid P (SAP), a 27 kDa protein that can bind to bacteria and cell debris. The binding of Fc

receptors to aggregated IgG or SAP leads to the engulfment of bacteria and debris, but whether this directly activates neutrophil is unknown.

4.2 Methods

4.2.1 Isolating peripheral blood mononuclear cells (PBMC) or neutrophils

PBMC were isolated and incubated in serum-free media SFM, consisting of FibroLife medium as described in chapter 2.2.1 (Shao et al., 2008). Neutrophils were isolated from blood using Lympholyte-poly (Cedarlane Laboratories, Hornby, Canada) following the manufacturer's directions and re-suspended in RPMI-1640 (Sigma) or 2%BSA (Sigma) in RPMI-1640.

4.2.2 Production of human SAP or murine SAP

Human SAP (hSAP) was from Calbiochem (Calbiochem-EMD Chemicals, Darmstadt, Germany). Commercial human SAP was buffer exchanged with 20 mM sodium phosphate buffer as described previously (Shao et al., 2008). Human SAP (hSAP) or murine SAP (mSAP) were also prepared from commercially available human serum (Gemini, West Sacramento, CA) or murine serum (Gemini) using calcium-dependent binding to phosphoethanolamine-conjugated agarose as described previously (Pilling et al., 2007). Commercial or purified SAP was kept at 1 mg/ml concentration in 20 mM sodium phosphate buffer, pH 7.4 at -20^{o}C.

4.2.3 Neutrophil spreading assay with cell debris

PBMC at 1 x 10^6 cells/ml in SFM were dounced with a drill and a Teflon douncing bit for 60 strokes to make cell debris. 100 µl of PBMC at 0.5 x 10^6 cells/ml were incubated in flat bottom 96-well tissue culture plates (BD, Franklin Lakes, NJ) in the presence or absence of 100 µl of undiluted debris for 7 days at 37^{o}C. On day 7, the supernatants were pipetted into eppendorf tubes and centrifuged at 10,000 x g for 10 minutes. Supernatants were again carefully pipetted out of the centrifuged eppendorf tubes and aliquoted into new eppendorf tubes. These eppendorf tubes containing PBMC supernatants were then flash frozen with liquid nitrogen, and stored at -80^{o}C until further use. 100 µl of 0.5 x 10^6 cells/ml neutrophils in RPMI were incubated with 50 µl of 40 µg/ml SAP in RPMI (from a 1 mg/ml stock of SAP in 20 mM sodium phosphate buffer, pH 7.4), and 50 µl RPMI, 50 µl of PBMC supernatant and 50 µl RPMI, 100 µl RPMI, or 50 µl of PBMC supernatant and 50 µl of 40 µg/ml SAP. After 1 hour, fields of neutrophils were photographed using a phase-contrast microscope with a 20 x objective.

4.2.4 Neutrophil adhesion assay on wet plasma fibronectin or cellular fibronectin

Wells of flat bottom 96-well tissue culture plates (BD) were pre-coated with 50 µl of 20 µg/ml bovine plasma fibronectin (Sigma) in PBS or 20 µg/ml cellular human foreskin fibroblast fibronectin (Sigma) in PBS for 1 hour at 37^{o}C. After removing the fibronectin, the wells were washed three times with 200 µl of PBS and then

blocked with 200 µl of 2% BSA-PBS for 2 hours at room temperature. The wells were then washed three times with 200 µl of PBS and once with 200 µl of 2% BSA-RPMI before adding neutrophils. 500 µl of neutrophils at 1 x 10^6 cells/ml in 2% BSA-RPMI were incubated in an eppendorf tube (pre-incubated with 2% BSA-RPMI for 2 hours at 37^{o}C) with 30 µg/ml SAP (from a 1 mg/ml stock made in 20 mM sodium phosphate buffer pH 7.4 of Calbiochem SAP or SAP purified from human serum) or an equal volume of buffer for 30 minutes at 37^{o}C immediately after isolating neutrophils. 100 µl of the neutrophils incubated with or without 30 µg/ml SAP was then incubated in the presence or absence of 1 µl of 10 µg/ml recombinant human-TNF-α (stock recombinant human-TNF-α was reconstituted to 100 µg/ml in water, 300-01, Peprotech, NJ) in 2% BSA-RPMI for 30 minutes at 37^{o}C in the well of a 96-well tissue culture plate. After a 30-minutes incubation with TNF-α, non-adherent neutrophils were removed and the wells were washed three times by pipetting in and then removing 100 µl of PBS. The plate was then air-dried, stained with methylene blue and eosin (Richard-Allan Scientific, Kalamazoo, MI) (Fischer et al., 2008), and the number of adherent neutrophils was counted in five different 900 µm diameter fields of view.

4.2.5 Neutrophil adhesion assay on dry cellular fibronectin

Wells of 96-well tissue culture plates were pre-coated with 60 µl of 20 µg/ml cellular fibronectin for 2 hours at room temperature. The fibronectin was then removed, and the wells were washed three times with 200 µl of PBS, and then

blocked with 1% BSA-PBS for 1 hour at room temperature. After blocking, BSA was removed, the wells were air-dried, and then washed three times with 100 µl of PBS per well. The wells were washed in 1% BSA-RPMI before adding neutrophils. Neutrophils at a concentration of 2 x 10^6 cells/ml in 1% BSA-RPMI were incubated in an eppendorf tube (pre-incubated with 1% BSA-RPMI for 2 hours at 37^{o}C) with 30 µg/ml SAP (from a 1 mg/ml stock made in 20 mM sodium phosphate buffer) or an equal volume buffer for 30 minutes at 37^{o}C. 50 µl of neutrophils incubated with or without 30 µg/ml SAP was then incubated in the presence or absence of 0.5 µl of 10 µg/ml TNF-α in 1% BSA-RPMI for 30 minutes at 37^{o}C in a 96-well tissue culture plate. After 30 minutes, non-adherent neutrophils were removed and the plates were washed three times by pipetting in and then removing 100 µl PBS, air-dried, stained, and the number of adherent neutrophils was counted.

4.2.6 Staining for neutrophil adhesion molecules

500 µl of neutrophils at 2.0 x 10^6 cells/ml were aliquoted into eppendorf tubes (pre-coated with 2% BSA-RPMI for 1 hour at 37^{o}C) and incubated in 10 ng/ml or 1 ng/ml TNF-α, 100 ng/ml IL-8, or 10 ng/ml or 1 ng/ml GM-CSF in the presence or absence of 10 µg/ml or 60 µg/ml SAP for one hour at 37^{o}C. For the neutrophils that were stained with (anti-human) anti-CD18, anti-CD61, or anti-CD44, 500 µl of neutrophils at 2.0 x 10^6 cells/ml were aliquoted into the eppendorf tubes (pre-coated with 2% BSA-RPMI for 1 hour at 37^{o}C) and treated

with 30 μg/ml SAP or equal volume buffer for 30 minutes at 37°C. Neutrophils were then divided into 3 eppendorf tubes (pre-coated with 2% BSA-RPMI for 1 hour at 37°C) and incubated for 30 minutes with 10 ng/ml TNF-α, 10 nM fMLP, or an equal volume buffer at 37°C. These neutrophils were then washed with ice-cold 1 x PBS and collected by centrifugation at 500 x g for 5 minutes. The supernatant was carefully removed, and the neutrophils were re-suspended in 1 ml of 4% BSA-PBS. Neutrophils from each condition were equally divided into new eppendorf tubes (pre-coated with 4% BSA-PBS for 1 hour at 4°C) and stained with 5 μg/ml primary antibodies against CD11b (Biolegend), CD62L (BD Biosciences), CD32 (BD Biosciences), CD18 (Biolegend), CD61 (BD Biosciences), CD44 (BD Biosciences), or mouse IgG1 isotype control (Biolegend), and then incubated with 2.5 μg/ml FITC-conjugated F(ab′)2 goat anti-mouse IgG antibodies (cross-adsorbed against human Ig, Southern Biotechnology, Birmingham, AL, USA) as described previously (Maharjan et al., 2010; Shao et al., 2008) and analyzed with a flow cytometer (Accuri Cytometers).

4.2.7 Quantifying the production of hydrogen peroxide

Wells of black 96-well cell culture plates (Nalge Nunc, Rochester, NY) were pre-coated with 50 μl of 20 μg/ml plasma fibronectin for 1 hour at 37°C. The fibronectin was then removed, and the wells were washed three times with 200 μl of PBS, and then washed once with Krebs-Ringer phosphate glucose (KRPG) buffer (145 mM NaCl, 4.86 mM KCl, 0.54 mM CaCl2, 1.22 mM MgSO4, 5.77 mM

sodium phosphate buffer, and 5.5 mM glucose, pH 7.35 (Nathan, 1987), before adding the neutrophils. 500 µl of neutrophils at a concentration of 15 x 10^5 cells/ml in KRPG buffer were incubated in an eppendorf tube (pre-incubated with 2% BSA-KRPG buffer for 2 hours at 37°C) with 30 µg/ml SAP (from a 1 mg/ml stock made in 20 mM sodium phosphate buffer) or an equal volume of buffer for 30 minutes at 37°C. An assay mixture of 100 µl of KRPG buffer, 20 µl of 300 µM scopoletin (Sigma), 20 µl of 10 mM NaN_3, and 20 µl of 10 U/ml horse radish peroxidase (Sigma) were aliquoted into a well and the plate was equilibrated to 37°C for 5 minutes as described previously (Delaharpe and Nathan, 1985; Nathan, 1987). 20 µl of neutrophils incubated with or without 30 µg/ml SAP was then added to the assay mixture in the presence or absence of 20 µl of 1 µg/ml TNF-α in KRPG buffer, 20 µl of 1 µM formyl-Met-Leu-Phe (fMLP) (Sigma) in KRPG buffer, 20 µl of 1 µM phorbol 12-myristate 13-acetate (PMA) (Sigma), 20 µl of 1 µM phorbol 12,13-dibutyrate (PDBu) (Sigma), or 20 µl of KRPG buffer. The 96-well plate was incubated at 37°C and the fluorescence (excitation: 360 nm emission: 460 nm) was monitored every 10 minutes for 3 hours using a Synergy MX plate reader (BioTek, Winooski, VT).

4.2.8 Transmigration assay of neutrophils

50 µl of neutrophils at 1 x 10^6 cells/ml in 2%BSA-RPMI was added on the top chamber of the 3 µm nylon membrane (BD) in the presence or absence of 10 nM fMLP, 30 µg/ml SAP, 10 nM fMLP and 30 µg/ml SAP, or an equal volume buffer

in 2% BSA-RPMI. The bottom chamber contained 600 µl of 10 nM fMLP, 30 µg/ml SAP, or 10 nM fMLP and 30 µg/ml SAP, or an equal volume buffer in 2% BSA-RPMI. The transmigration was carried out for 2 hours at 37^{o}C. The top chamber was removed and 25 µl of the neutrophils that had migrated into the bottom chamber was then counted with a flow cytometer (Accuri Cytometers).

4.2.9 Murine neutrophil adhesion assay

Mice were euthanized and blood was obtained via cardiac puncture. 2-4 ml of blood was collected in an EDTA-containing vacutainer tube (BD) and the red blood cells (RBC) in 2 ml of blood were lysed by adding 1 ml of ammonium chloride, potassium bicarbonate (ACK) lysis buffer (15 mM NH_4Cl, 1 mM $KHCO_3$, 0.01 mM Na_2EDTA) and incubating for 3 minutes at room temperature. Eppendorf tubes containing blood and ACK lysis buffer were centrifuged at 500 x g for 5 minutes at room temperature, and the supernatant containing lysed RBC was removed. The pellets were re-suspended in 200 µl PBS, and 1 ml ACK lysis buffer was added. After three minutes, cells were collected by centrifugation. This was then repeated two additional times. Cells were re-suspended in 1 ml PBS and then collected by centrifugation. The cells were then re-suspended in 1 ml of 2% BSA-RPMI. A neutrophil adhesion assay was carried out in 2% BSA-RPMI similar to the human neutrophil adhesion assay using 60 µg/ml human SAP instead of 30 µg/ml. The adhered cells were stained for Ly6G to distinguish neutrophils from other cell types as described previously

(Pilling et al., 2009). The number of adhered Ly6G-positive neutrophils was then counted as described above.

4.2.10 Animal model of acute respiratory distress syndrome (ARDS)

ARDS-like pathology was induced in 4 weeks old C57/BL6 mice (The Jackson Laboratory, Bar Harbor, ME) with an oropharyngeal aspiration of 50 μl of 0.2 U/kg or 3 U/kg bleomycin (Calbiochem) (Pilling et al., 2009). Mice were anesthetized and laid on a 60^{o} inclined board. The mouth of the mouse was opened using a thin rubber band that held the upper front teeth of the mouse. During oropharyngeal aspiration, the nares of the mouse were pinched shut with a pair of forceps and the tongue was gently extracted out with another pair of forceps as described previously (Lakatos et al., 2006). With a pipettor, bleomycin was quickly aspirated through the mouth of the mouse. The successful aspiration of bleomycin into the lungs was confirmed by listening to the crackling noise heard after the aspiration. The mice were used in accordance with guidelines published by the National Institutes of Health, and the protocol was approved by the Texas A&M University Animal Use and Care Committee.

4.2.11 Injections of SAP

At 24 and 48 hours following bleomycin aspiration (days 1 and 2), C57/BL6 or *sap*$^{-/-}$ mice were treated with an intraperitoneal injection of 50 μl of 1 mg/ml hSAP or mSAP in 20 mM sodium phosphate buffer or an equal volume of 20 mM sodium phosphate buffer.

4.2.12 Quantification of different types of cells

Mice were euthanized at day 3 after bleomycin aspiration, and the lungs were perfused with 400 µl of PBS for three times to collect cells by bronchoalveolar lavage (BAL) as described previously (Corteling et al., 2002). The cells were collected by centrifugation at 500 x g for 5 minutes, and the supernatants were transferred to eppendorf tubes. The pooled supernatants were flash frozen with liquid nitrogen, and stored at -80^{o}C until further use. The cells collected from BAL were re-suspended in 100 µl of 4%BSA-PBS and counted with a hemacytometer. The cells were then diluted in a total volume of 600 µl of 4% BSA-PBS. 100 µl of diluted cells were then aliquoted into cytospin funnels and were then spun onto glass slides (Superfrost plus white slides, VWR, West Chester, PA) at 400 x g for 5 minutes using a cytospin centrifuge (Shandon Southern Products Ltd, Cheshire, England). These cells were then air-dried, and stained with 5 µg/ml anti-mouse Ly6G (Biolegend, San Diego, CA) as previously described (Pilling et al., 2009). After staining the cells, the number of cells positive for Ly6G per 200 cells was counted. The percent of positive cells was then multiplied by the total number of cells recovered from the BAL to obtain the number of neutrophils in the BAL.

4.2.13 Immunohistochemistry

After BAL, lungs were inflated with pre-warmed OCT (VWR) and then embedded in OCT, frozen on dry ice, and stored at -80^{o}C as described previously (Pilling et al., 2007). Lung tissue sections (6 µm) were prepared and immunohistochemistry was done as described previously (Pilling et al., 2007) except slides were incubated with 2.5 µg/ml primary antibodies in 4% BSA-PBS for 60 minutes. The lung sections were stained for Ly6G (BioLegend) to detect neutrophils, CD11b (BioLegend) to detect macrophages, and CD45 (BioLegend) to detect all leukocytes. Isotype-matched mouse irrelevant antibodies were used as controls. Slides were then washed three times with PBS over 30 minutes and incubated with 1.25 µg/ml biotinylated mouse F(ab')2 anti-rat IgG in 4% BSA-PBS for 30 minutes. Slides were then washed three times in PBS over 30 minutes and incubated with a 1:500 dilution of streptavidin alkaline phosphatase (Vector Laboratories) in 4% BSA-PBS for 30 minutes. Staining was developed with a VectorRed Alkaline Phosphatase Kit (Vector Laboratories) for 10 minutes. Slides were then mounted on permount as described previously.

4.2.14 Statistics

Statistical analysis was performed using Prism (GraphPad Software, San Diego, CA, USA). Statistical significance was determined using either analysis of variance (ANOVA) or t-test, and significance was defined as $p < 0.05$.

4.3 Results

4.3.1 SAP inhibits neutrophil spreading induced by PBMC conditioned media

Factors such as bacteria and cell debris elicit the release of different factors that signal neutrophil to spread, adhere, and migrate to the sites of infection or injury (Perl et al., 2008). We previously found that a protein in plasma called serum amyloid P (SAP) inhibits the differentiation of monocytes to fibrocytes (Pilling et al., 2003). Since neutrophils have Fcγ receptors that can bind to proteins such as serum amyloid P (SAP), we examined the effect of SAP on neutrophil spreading. To determine the role of SAP on neutrophil spreading, David Roife and Dr. Gomer incubated human PBMC with cell debris for 7 days at 37^{o}C. On the 7th day, they removed the conditioned media (which should contain cell debris as well as PBMC-derived signals elicited in response to debris) and incubated freshly isolated human neutrophils in the presence or absence of 10 μg/ml SAP, PBMC conditioned media, or the combination of PBMC conditioned media and 10 μg/ml SAP. They observed that there were neutrophils that were spreading in the control experiment, which could have been an effect of laying neutrophils directly on a plastic tissue culture plate (Figure 4.3.1). When neutrophils were incubated with 10 μg/ml SAP, the neutrophils had a rounded morphology and decreased neutrophil spreading (Figure 4.3.1). This suggests SAP is directly acting on the neutrophils to inhibit the spreading. When neutrophils were incubated with PBMC conditioned media, there were increased numbers of neutrophils that were spreading (Figure 4.3.1). However, when

neutrophils were incubated with the combination of PBMC conditioned media and 10 µg/ml SAP, SAP significantly inhibited the spreading of neutrophils induced by the PBMC conditioned media (Figure 4.3.1). Overall, these results suggest that SAP inhibits neutrophil spreading.

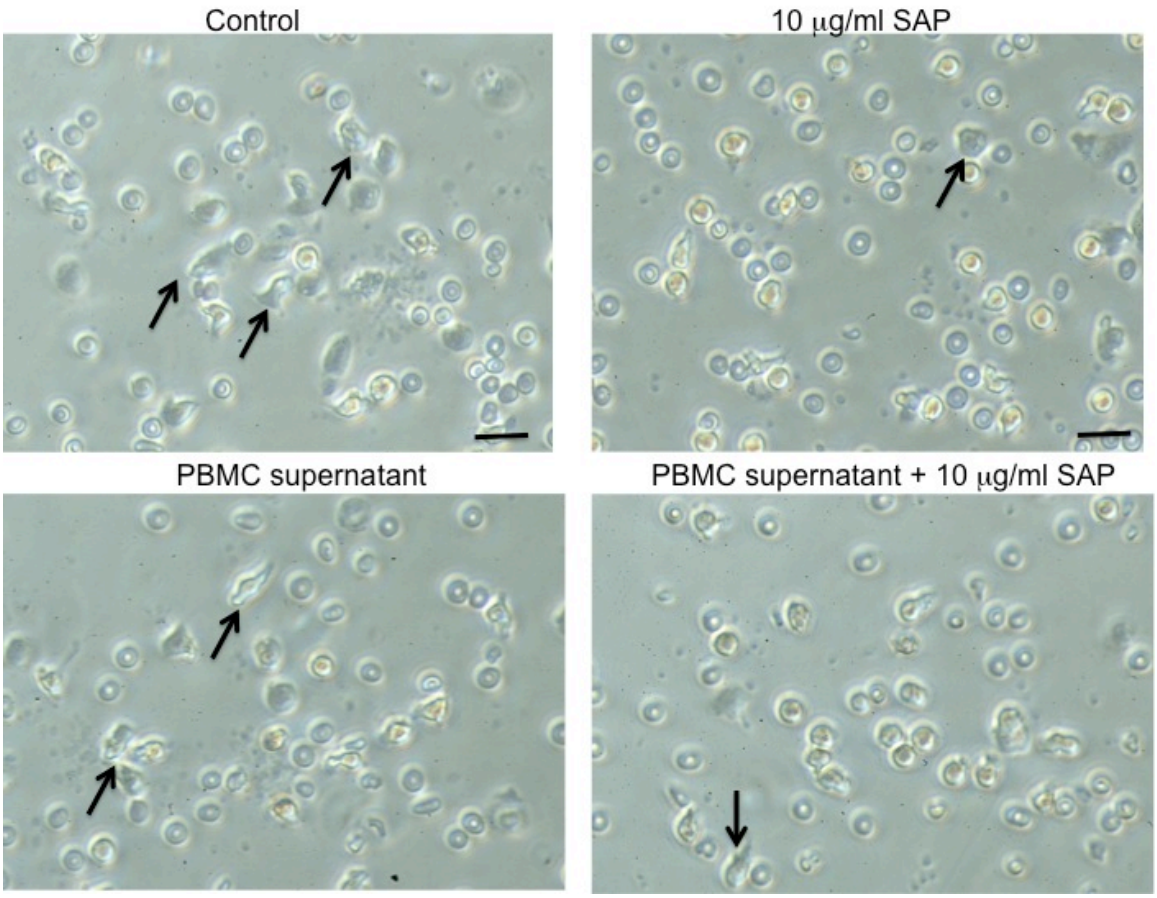


Figure 4.3.1: SAP inhibits neutrophil spreading. Human PBMC were isolated and incubated in the presence of cell debris for 7 days at 37°C. On the 7th day, the conditioned media was removed. Human neutrophils were incubated in the presence or absence of 10 μg/ml human SAP (hSAP), PBMC conditioned media, or the combination of PBMC conditioned media for 1 hour at 37°C. Fields of neutrophils were photographed using a phase-contrast microscope with a 20 x objective at 1 hour. Arrows indicate spread neutrophils. All of the figures represent one of 3 separate experiments. Bar is 20 μm. Figure is from Dr. Richard H. Gomer and David Roife.

4.3.2 SAP inhibits neutrophil adhesion induced by TNF-α

TNF-α increases the adherence of neutrophils on a variety of extracellular matrices (Kettritz et al., 2004; Nathan, 1987). Since we observed that SAP inhibits neutrophil spreading, we wanted to determine the role of SAP in neutrophil adhesion. Freshly isolated neutrophils were incubated with or without 30 μg/ml of SAP for 30 minutes at 37°C. These neutrophils were then incubated with 100 ng/ml TNF-α for 30 minutes at 37°C in plates pre-coated with BSA, plasma fibronectin, or cellular fibronectin. Neutrophils incubated with TNF-α had increased number of neutrophils that adhered to the wells (Figure 4.3.2A-C). When the 96 well plates were coated with BSA or cellular fibronectin, neutrophils treated with 30 μg/ml SAP had no significant change in neutrophil adherence when compared to the control (Figure 4.3.2A and 4.3.2C). However, when the 96 well plates were coated with plasma fibronectin, treatment of neutrophils with 30 μg/ml SAP decreased the number of adhered neutrophils compared to the control (Figure 4.3.2B). When neutrophils pre-treated with SAP were incubated with 100 ng/ml TNF-α, SAP significantly inhibited the TNF-α-induced adhesion of neutrophils to BSA, plasma fibronectin, and cellular fibronectin (Figure 4.3.2A-C). Similarly, Dr. Derrick Brazilis also found that SAP pre-treatment inhibited neutrophil adhesion induced by TNF-α in 96 well culture plates that had been pre-coated with cellular fibronectin and had been air-dried before adding

neutrophils (Figure 4.3.3). These results suggest SAP inhibits neutrophil adhesion induced by TNF-α.

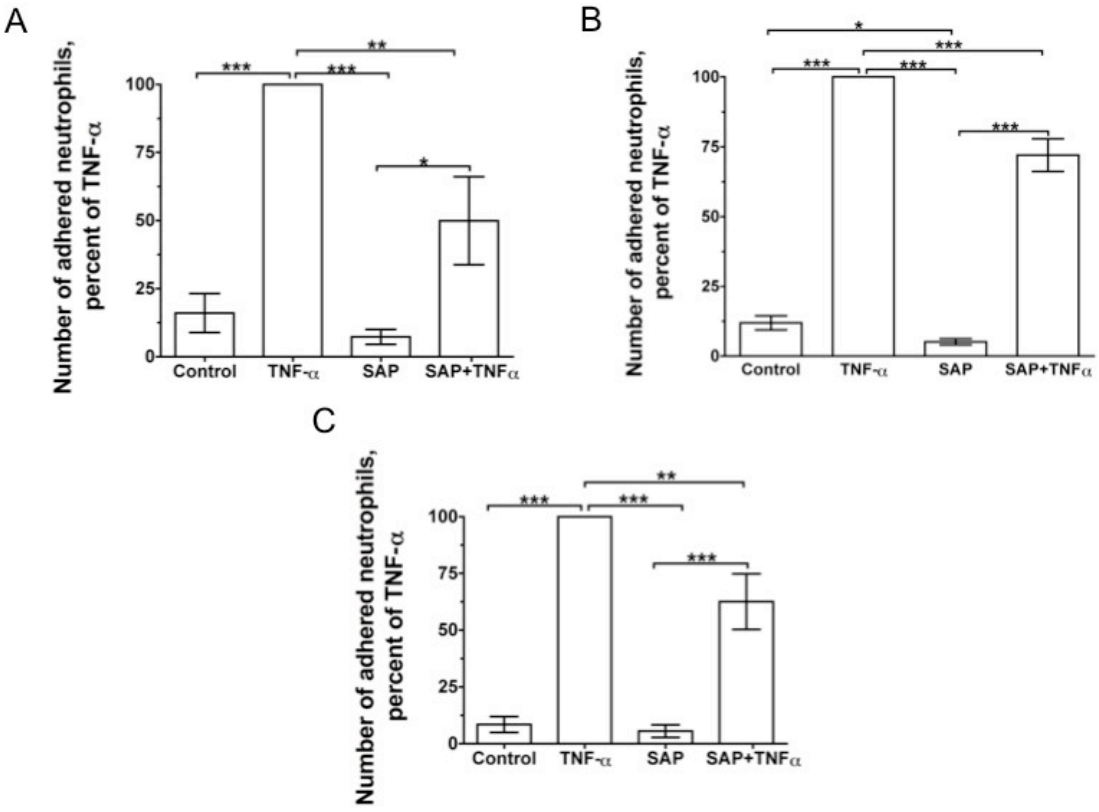


Figure 4.3.2. SAP inhibits neutrophil adhesion induced by TNF-α. **(**A) 96-well tissue culture plates were blocked with 2% BSA-PBS for 2 hours at room temperature. The plates were washed in PBS, and once with 2% BSA-RPMI. Human neutrophils were pre-treated with or without 30 µg/ml hSAP for 30 minutes at 37°C. 100 µl of 1 x 10^6 cell/ml neutrophils were seeded on the plate, and 1 µl of 10 µg/ml recombinant human-TNF-α was added to the neutrophils for another 30 minutes at 37°C. After removing the media and washing the plates, the plates were air-dried, fixed, stained, and the number of neutrophils that remained in the wells was counted (n = 4). (B) 96-well tissue culture plates were

pre-coated with 20 μg/ml plasma fibronectin and then blocked with 2% BSA-PBS for 2 hours at room temperature. Neutrophils were incubated in the presence or absence of 30 μg/ml SAP or 100 ng/ml TNF-α and counted as described above (n = 23). Using two-tailed t-test, SAP significantly inhibited neutrophil adhesion when compared to the control with $p < 0.05$. (C) 96-well tissue culture plates were pre-coated with 20 μg/ml cellular fibronectin and then blocked with 2% BSA-PBS for 2 hours at room temperature. Neutrophils were incubated in the presence or absence of 30 μg/ml SAP or 100 ng/ml TNF-α and counted as described above (n = 5). When the number of neutrophils treated with TNF-α, SAP, SAP and TNF-α, or control was normalized to the number of neutrophils treated with TNF-α, there was a significant difference in the number of adhered neutrophils (1-way ANOVA, Tukey's test). ** indicates $p < 0.01$, and *** indicates $p < 0.001$.

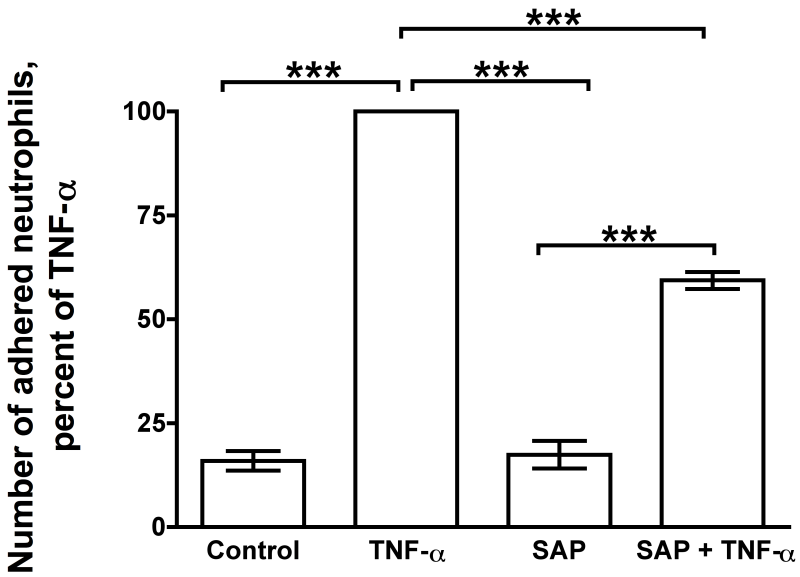


Figure 4.3.3: SAP inhibits neutrophil adhesion induced by TNF-α on dry fibronectin. 96-well tissue culture plates were pre-coated with 20 µg/ml cellular fibronectin and then blocked with 2% BSA-PBS for 2 hours at room temperature. The plate was then air-dried. Human neutrophils were pre-incubated in the presence or absence of 30 µg/ml SAP, plated on the 96-well tissue culture plate, and stimulated with 100 ng/ml TNF-α for 30 minutes at 37°C. Non-adhered neutrophils were removed and adhered neutrophils were counted as described in Figure 4.3.2 (n = 3). When the number of neutrophils treated with TNF-α, SAP, SAP and TNF-α, or control was normalized to the number of neutrophils treated with TNF-α, there was a significant difference in the number of adhered neutrophils (1-way ANOVA, Tukey's test). *** indicates $p < 0.001$. Figure is from Dr. Derrick Brazilis.

4.3.3 SAP inhibits murine neutrophil adhesion induced by TNF-α

Since we found that human SAP can inhibit human neutrophil adhesion induced by TNF-α, we wanted to determine if human SAP could also inhibit murine neutrophil adhesion induced by TNF-α. Freshly isolated murine neutrophils were incubated with or without 60 μg/ml of human SAP for 30 minutes at 37°C. These neutrophils were then incubated with 100 ng/ml TNF-α for 30 minutes at 37°C in plates pre-coated with plasma fibronectin. There was an increased number of neutrophils that adhered to the wells when neutrophils were treated with TNF-α (Figure 4.3.4). Neutrophils treated with 60 μg/ml SAP had no significant change in neutrophil adherence when compared to the control (Figure 4.3.4). However, when neutrophils pre-treated with SAP were incubated with 100 ng/ml TNF-α, SAP significantly inhibited the TNF-α-induced adhesion of neutrophils to plasma fibronectin (Figure 4.3.4). These results suggest that human SAP inhibits murine neutrophil adhesion induced by TNF-α.

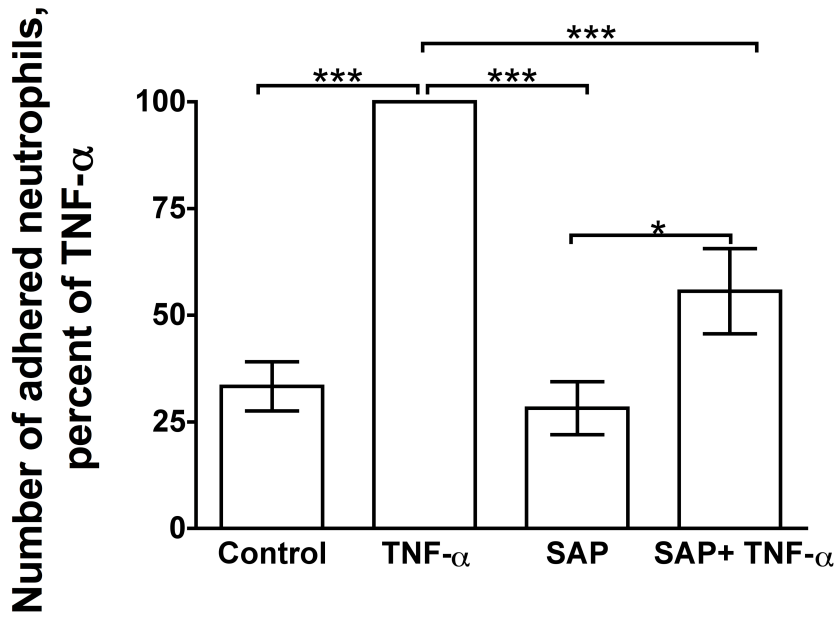


Figure 4.3.4 Human SAP inhibits murine neutrophil adhesion induced by TNF-α. 96-well tissue culture plates were pre-coated with 20 μg/ml plasma fibronectin and then blocked with 2% BSA-PBS for 2 hours at room temperature. Murine blood was lysed with ACK lysis buffer and isolated cells were incubated in the presence or absence of 60 μg/ml SAP for 30 minutes at 37°C. Cells were then plated on a 96-well tissue culture plate in the presence or absence of 100 ng/ml TNF-α for 30 minutes at 37°C. Non-adhered cells were removed, and adhered cells were stained for Ly6G (a marker for murine neutrophils). The number of adhered neutrophils was counted as described in Figure 4.3.2 (n = 8). There was a significant difference in the number of adhered neutrophils (1-way ANOVA, Tukey's test). * indicates $p < 0.05$ and ** indicates $p < 0.01$.

4.3.4 SAP has no effect on adhesion molecules induced by TNF-α or GM-CSF

To determine how SAP decreases the number of adherent neutrophils in the neutrophil plate adhesion assay, I analyzed receptors whose levels change in activated neutrophils. CD11b are adhesion molecules that help neutrophils to extravagate into injured sites, while CD62L help neutrophils to roll inside blood vessels when the cells are in a quiescent phase (Hughes et al., 2007; Taylor et al., 2007a). CD32 are the major Fc receptors of neutrophils (Tsuboi et al., 2008). Neutrophils were treated with 10 ng/ml or 1 ng/ml TNF-α, 100 ng/ml IL-8, or 10 ng/ml or 1 ng/ml GM-CSF in the presence or absence of 10 μg/ml or 60 μg/ml SAP for one hour at 37°C. After the reaction was stopped, the cells were stained for CD11b, CD32, and CD62L. When neutrophils were treated with 10 ng/ml or 1 ng/ml TNF-α , 100 ng/ml IL-8, or 10 ng/ml or 1 ng/ml GM-CSF in the presence or absence of 10 μg/ml or 60 μg/ml SAP, there was no significant effect on the number of CD11b-positive cells (Figure 4.3.5A). However, the staining intensity of CD11b was different in various conditions (Figure 4.3.6A). IL8 in the presence or absence of SAP had no effect on the levels of CD11b or CD62L compared to untreated neutrophils (Figure 4.3.6A). TNF-α or GM-CSF treated neutrophils with or without SAP had increased levels of CD11b compared to other conditions (Figure 4.3.6A). This suggests that TNF-α and GM-CSF activate neutrophils, and that the addition of SAP has no effect on the activation. When neutrophils were treated with 10 ng/ml or 1 ng/ml TNF-α or 10 ng/ml or 1 ng/ml GM-CSF in

the presence or absence of 60 µg/ml SAP, there was a decrease in the number of CD62L-positive cells in these conditions (Figure 4.3.5B). Cells treated with TNF-α or GM-CSF in the presence or absence of SAP had decreased levels of CD62L compared to other conditions (Figure 4.3.6A). This again suggests that TNF-α and GM-CSF activate neutrophils, and that the addition of SAP has no effect on the activation. There was no significant effect on the levels of CD32 or mouse IgG1 (control) staining when neutrophils were treated with 10 ng/ml TNF-α or 10 ng/ml IL-8 in the presence or absence of 60 µg/ml SAP (Figure 4.3.5C, 4.3.5D, and 4.3.6B). TNF-α and GM-CSF increased the levels of CD11b and decreased the levels of CD62L-positive cells. This suggests that TNF-α and GM-CSF activate neutrophils (Cassatella, 1999; Montecucco et al., 2008; Yong and Linch, 1992), and that the addition of SAP has no effect on the activation.

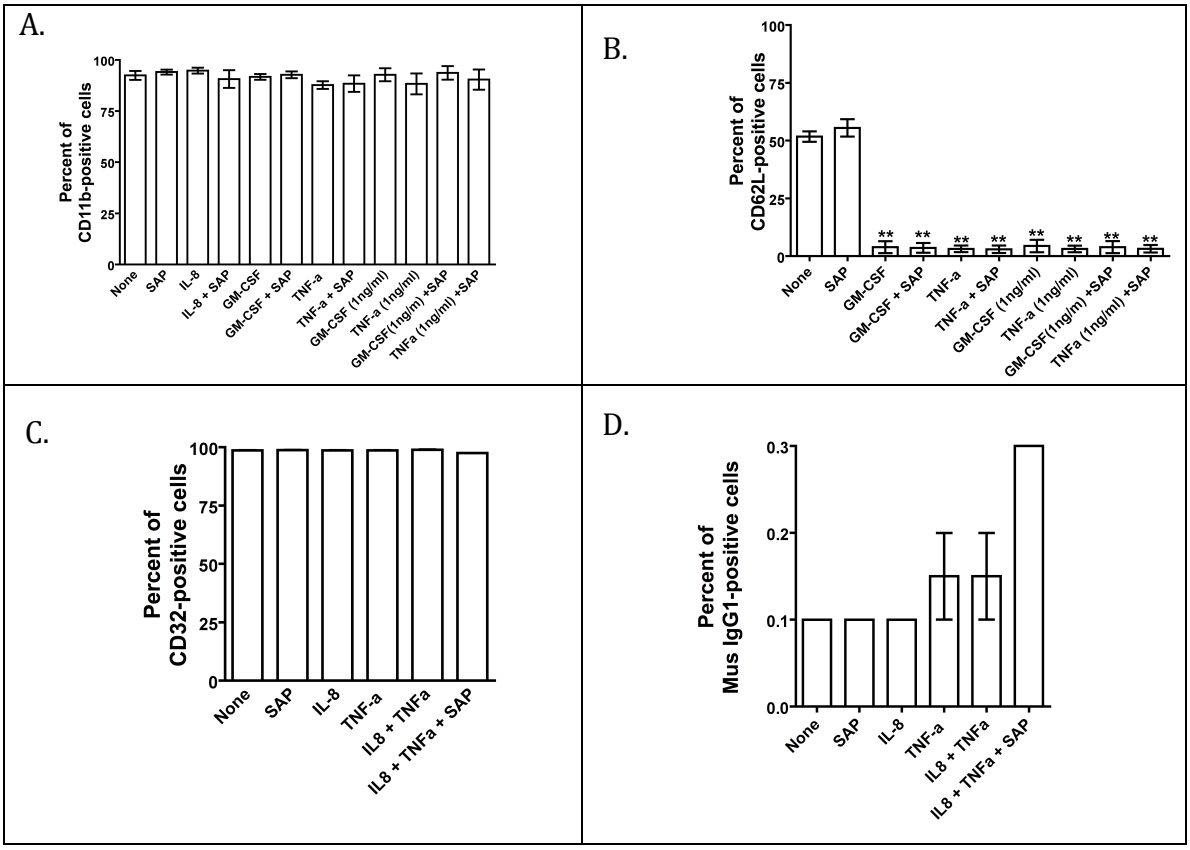


Figure 4.3.5: SAP has no effect on the number of surface receptors associated with neutrophil activation. (A) Human neutrophils were treated with 100 ng/ml IL-8, 10 ng/ml or 1 ng/ml GM-CSF, or 10 ng/ml or 1 ng/ml TNF-α in the presence or absence of SAP (10 µg/ml for IL-8 and 60 µg/ml for GM-CSF and TNF-α) for 1 hour at 37^{o}C. The cells were then stained for CD11b. The results are mean ± SEM of percent control of CD11b-positive cells (n = 3 separate experiments). (B) Neutrophils were treated with GM-CSF (10 ng/ml or 1 ng/ml) or TNF-α (10 ng/ml or 1 ng/ml) in the presence or absence of SAP (60 µg/ml) for 1 hour at 37^{o}C. The cells were then stained for CD62L. The results are mean ± SEM of percent control of CD62L-positive cells (n = 3 separate experiments). (C) Neutrophils

were treated with IL-8 (100 ng/ml) or TNF-α (10 ng/ml) in the presence or absence of SAP (10 μg/ml) for 1 hour at 37^{o}C. The cells were then stained for CD32. The results are mean ± SEM of percent control of CD32-positive cells (n = 2 separate experiments). (D) Neutrophils were treated with the above conditions as in Fig. 4.3.5C for 1 hour at 37^{o}C. The cells were then stained with mouse IgG1. The results are mean ± SEM of percent control of mouse IgG1-positive cells (n = 2 separate experiments).

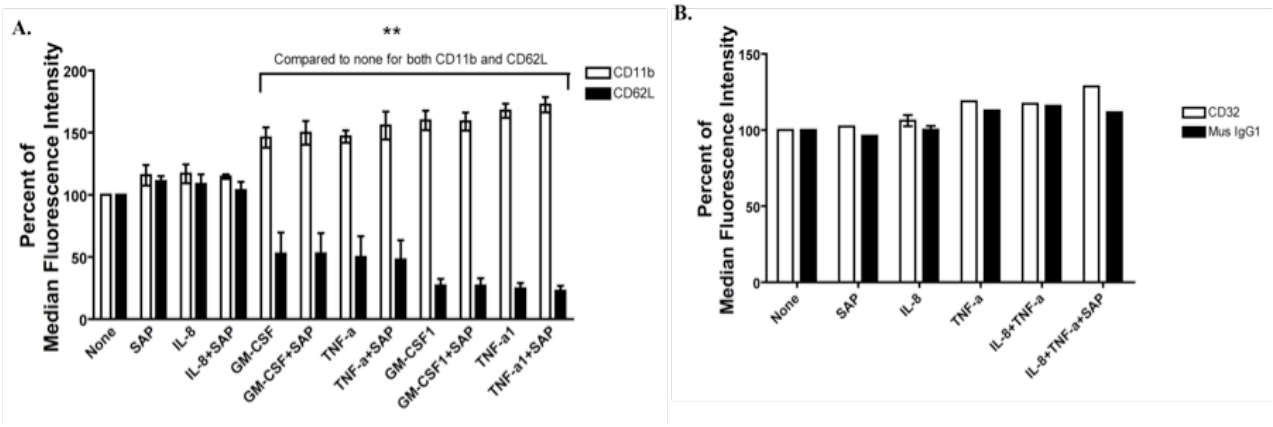


Figure 4.3.6: SAP has no effect on the levels of CD11b, CD62L, or CD32. (A) Human neutrophils were treated with the above conditions as in Fig. 4.3.5A for 1 hour at 37°C. The cells were then stained for CD11b or CD62L. The results are mean ± SEM of percent control of median fluorescence intensity of CD11b- or CD62L-positive cells (n = 3 separate experiments except n = 2 for IL-8 and IL-8+SAP). (B) Neutrophils were treated with the above conditions as in Fig. 4.3.5C for 1 hour at 37°C. The cells were then stained for CD32 or stained with a control mouse IgG1. The results are mean ± SEM of percent control of median fluorescence intensity of CD32- or mouse IgG1-positive cells (n = 2 separate experiments).

4.3.5 SAP has no effect on the levels of the surface receptors CD18, CD61, or CD44.

Since SAP had no effect on the levels of the surface receptors CD11b or CD62L, I examined the levels of other adhesion molecules such as CD18, CD61, or CD44. CD18 is an integrin molecule that binds to CD11b and aids in leukocyte migration (Johnson and Ruffell, 2009). CD61 is also an integrin molecule that facilitates leukocyte migration (Johnson and Ruffell, 2009), but little is known about its role in neutrophil migration. CD44 is a cell surface adhesion receptor that also helps in leukocyte migration (Johnson and Ruffell, 2009), but again little is known about its role in neutrophil migration. Freshly isolated human neutrophils were treated with or without 30 μg/ml SAP for 30 minutes at 37^{o}C. After 30 minutes, 10 ng/ml TNF-α or 10 nM formyl-Met-Leu-Phe (fMLP) was added to cells with or without SAP and incubated for another 1 hour at 37^{o}C. Neutrophils were removed and stained for CD18, CD61, or CD44. Neutrophils treated with TNF-α showed increased levels of the surface receptor CD18 and decreased levels of the surface receptor CD44 (Figure 4.3.7). The levels of CD61 did not change with the addition of TNF-α. SAP had no effect on the levels of the surface receptors CD18, CD61, or CD44. Neutrophils treated with TNF-α and SAP showed increased levels of CD18 and decreased levels of CD44. The levels of CD61 did not change with the addition of TNF-α or SAP. Neutrophils treated with fMLP showed a slight increase in the levels of CD18, but there was no change with the levels of CD61or CD44. Neutrophils treated with fMLP and SAP also showed a slight increase in the levels of CD18, but there was no

change with the levels of CD61, or CD44. Adding SAP to neutrophils treated with TNF-α or fMLP had no effect on the levels of the surface receptors CD18, CD61, or CD44.

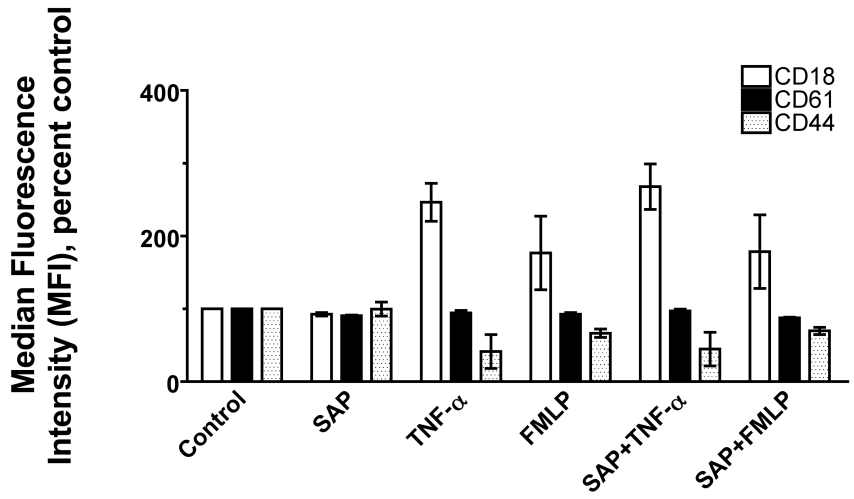


Figure 4.3.7: SAP has no effect on the levels of CD18, CD 61, or CD44. Neutrophils pre-treated with or without SAP were incubated in the presence or absence of TNF-α (10 ng/ml) or fMLP (10 nM) for 1 hour at 37^{o}C. The cells were then stained for anti-human CD18, anti-human CD61, or anti-human CD44. The results are mean ± SEM of median fluorescence intensity of positively stained cells (n = 3 separate experiments for CD18 and CD61 and n = 2 separate experiments for CD44).

4.3.6 In neutrophils, SAP has no effect on the production of hydrogen peroxide induced by TNF-α

Activated neutrophils release reactive oxygen species such as hydrogen peroxide and superoxide anions through NADPH oxidase (Waddell et al., 1994). The enzyme produces reactive oxygen species to kill microbes such as bacteria. However, excessive release of these cytotoxic products can further damage an injured tissue. To examine whether SAP can inhibit the production of hydrogen peroxide induced by TNF-α, fMLP, PDBu, or PMA, we examined the change in the fluorescence intensity of scopoletin, a fluorescent molecule that gets modified by hydrogen peroxide. I used the fluorescence arbitrary unit to obtain hydrogen peroxide produced per 1.5 x 10^4 cells as previously described (Nathan et al., 1989; Nathan, 1987). There was an increase in the production of hydrogen peroxide when cells were treated with TNF-α, fMLP, PDBu, or PMA (Figure 4.3.8). SAP had no effect on the production of hydrogen peroxide induced by TNF-α (Figure 4.3.8A), fMLP (Figure 4.3.8B), PDBu (Figure 4.3.8C), or PMA (Figure 4.3.8D).

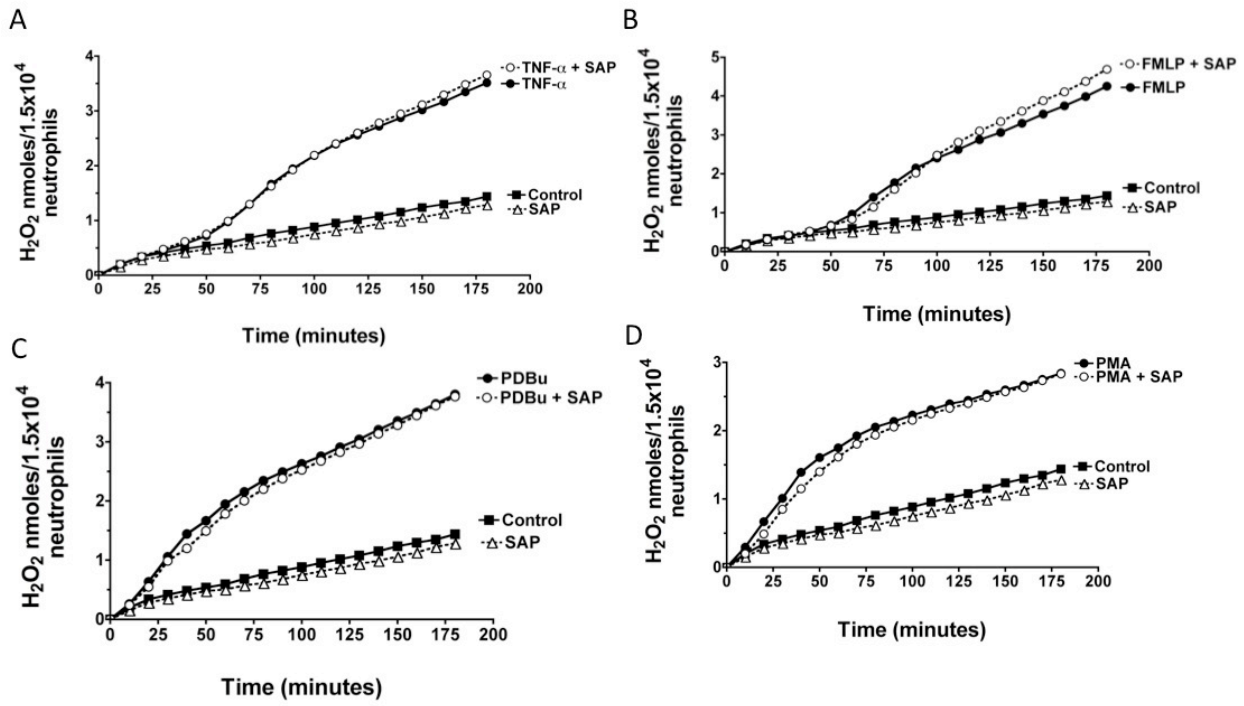


Figure 4.3.8: SAP has no effect on the production of hydrogen peroxide induced by TNF-α, PMA, PDBu, or FMLP. **(**A) Neutrophils pre-treated with 30 µg/ml SAP were treated with 10 ng/ml TNF-α for 3 hours at 37°C. Fluorescence intensity was measured every 10 minutes. (B) Neutrophils pre-treated with 30 µg/ml SAP were treated with 10 nM fMLP for 3 hours at 37°C. The results are hydrogen peroxide produced in different condition every 10 minutes for 3 hours. (C) Neutrophils pre-treated with 30 µg/ml SAP were treated with 10 nM PDBu for 3 hours at 37°C. (D) Neutrophils pre-treated with 30 µg/ml SAP were treated with 10 nM PMA for 3 hours at 37°C. (All of the figures represent one of the 2 separate experiments).

4.3.7 SAP has no effect on the migration of neutrophils.

The formyl peptide fMLP induces the migration of neutrophils (Quinn et al., 2007). To determine the role of SAP in neutrophil migration, I carried out migration assays using a Boyden chamber. Neutrophils are placed on a porous membrane and the bottom of the membrane touches a solution containing buffer or fMLP in the presence or absence of SAP. Migration was carried out for 2 hours at 37^{o}C and 25 µl of cells from the bottom chamber was counted with a flow cytometer. fMLP significantly increased the number of neutrophils that migrated across the porous membrane, while SAP had no effect on the migration of neutrophils (Figure 4.3.9). SAP also had no affect on the migration of neutrophils caused by fMLP (Figure 4.3.9).

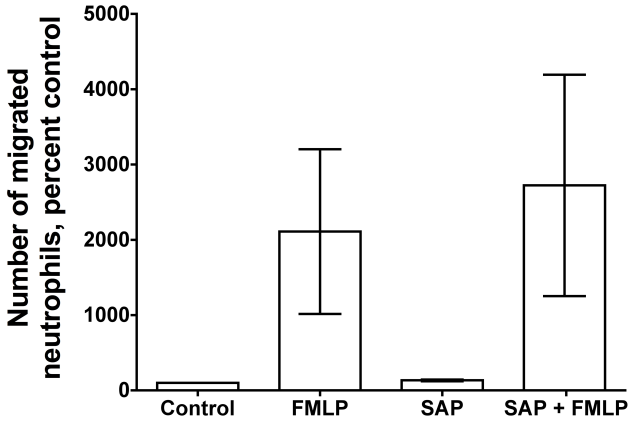


Figure 4.3.9: SAP has no effect on the migration of neutrophils. Neutrophils were placed on a porous membrane that touched a chamber containing buffer, 10 nM FMLP, 30 µg/ml SAP, or 10 nm fMLP and 30 µg/ml SAP. Migration was carried out for 2 hours at 37^{o}C. 25 µl of neutrophils from the bottom chamber were counted with a flow cytometer. The results are mean ± SEM of migrated neutrophils (n = 4 separate experiments).

4.3.8 SAP inhibits the accumulation of Ly6G-positive cells in lungs of mice treated with bleomycin

In acute lung injury and acute respiratory distress syndrome (ARDS), there is a massive accumulation of neutrophils in the lungs that further worsens the condition by producing superoxide anions and hydrogen peroxide (Lee and Downey, 2001; Perl et al., 2008). Since SAP inhibits neutrophil adhesion, we examined the effect of SAP on bleomycin-induced neutrophil accumulation in the lungs of mice. Mice were treated with or without 0.2 U/kg or 3 U/kg bleomycin on day 0 and then given intraperitoneal injections of 50 μg SAP in 20 mM sodium phosphate buffer on days 1 and 2. On day 3, the mice were euthanized and cells from the lungs were collected by BAL. There was no statistically significant difference in the number of cells collected from BAL of control, saline, 0.2 U/kg bleomycin and buffer, 0.2 U/kg bleomycin and human SAP, or 0.2 U/kg bleomycin and mouse SAP (Figure 4.3.10A). Cells collected from the BAL of mice were then stained with anti-mouse Ly6G (this stains neutrophils) (Lai et al., 1998; Landsman et al., 2007) (Figure 4.3.10B). There was an increased number of Ly6G-positive cells in the BAL from mice treated with 0.2 U/kg bleomycin and buffer compared to the number of Ly6G-positive cells in the BAL from control (untreated mice) or saline-treated mice (Figure 4.3.10B). There was no significant difference between the number of Ly6G-positive cells in the BAL from mice treated with 0.2 U/kg bleomycin and human SAP or 0.2 U/kg bleomycin and mouse SAP when compared to control (untreated mice) or saline-treated mice.

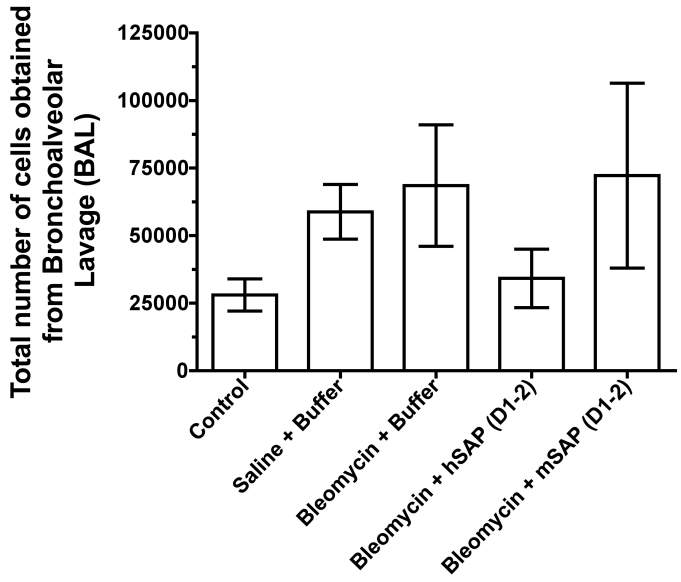
However, there was a decreased number of Ly6G-positive cells in the BAL from mice treated with 0.2 U/kg bleomycin and human SAP or 0.2 U/kg bleomycin and mouse SAP when compared to the BAL from mice treated with 0.2 U/kg bleomycin and buffer (Figure 4.3.10B).

We also treated mice with 3 U/kg bleomycin, followed by intraperitoneal injections of 50 µl of buffer containing 50 µg human SAP, or 50 µl of buffer, after 24 and 48 hours. There was no statistically significant difference between the total number of cells collected from the BAL of control or saline-treated mice (Figure 4.3.11A). However, there was an increased number of total cells collected in the BAL from mice treated with 3 U/kg bleomycin and buffer or 3 U/kg bleomycin and human SAP when compared to the total cells collected in the BAL from control or saline-treated mice (Figure 4.3.11A). There was also an increased number of Ly6G-positive cells in the BAL from mice treated with 3 U/kg bleomycin and buffer when compared to the number of Ly6G-positive cells in the BAL from control or saline-treated mice (Figure 4.3.11B). However, there was no statistically significant difference in Ly6G-positive cells in the BAL from mice treated with 3 U/kg bleomycin and human SAP when compared to the Ly6G-positive cells in the BAL from control or saline-treated mice (Figure 4.3.11B). There was a decreased number of Ly6G-positive cells in the BAL from mice treated with 3 U/kg bleomycin and human SAP when compared to the number of

Ly6G-positive cells in the BAL from mice treated with 3 U/kg bleomycin and buffer (Figure 4.3.11B).

We further stained lung sections with Ly6G after BAL to detect neutrophils remaining in the lungs after BAL. For both 0.2 and 3 U/kg bleomycin followed by buffer injections, there were more neutrophils observed in the lung sections compared to the lung sections from other treatment groups (Figures 4.3.10C and 4.3.11C). This indicates that if we had been able to obtain all of the lung neutrophils, the difference between bleomycin/buffer injections and bleomycin/SAP injections would have been even greater than the differences shown in Figure 4.3.10B and 4.3.11B. Our results thus suggest that SAP reduces the bleomycin-induced accumulation of neutrophils in the lungs of mice.

A


Total number of cells obtained from Bronchoalveolar Lavage (BAL)
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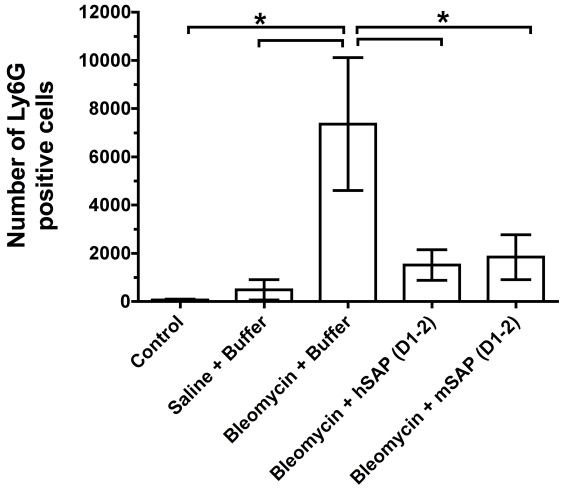
100000
75000
50000
25000
0
Control
Saline + Buffer
Bleomycin + Buffer
Bleomycin + hSAP (D1-2)
Bleomycin + mSAP (D1-2)

B


Number of Ly6G positive cells
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10000
8000
6000
4000
2000
0
*
*
Control
Saline + Buffer
Bleomycin + Buffer
Bleomycin + hSAP (D1-2)
Bleomycin + mSAP (D1-2)

C.

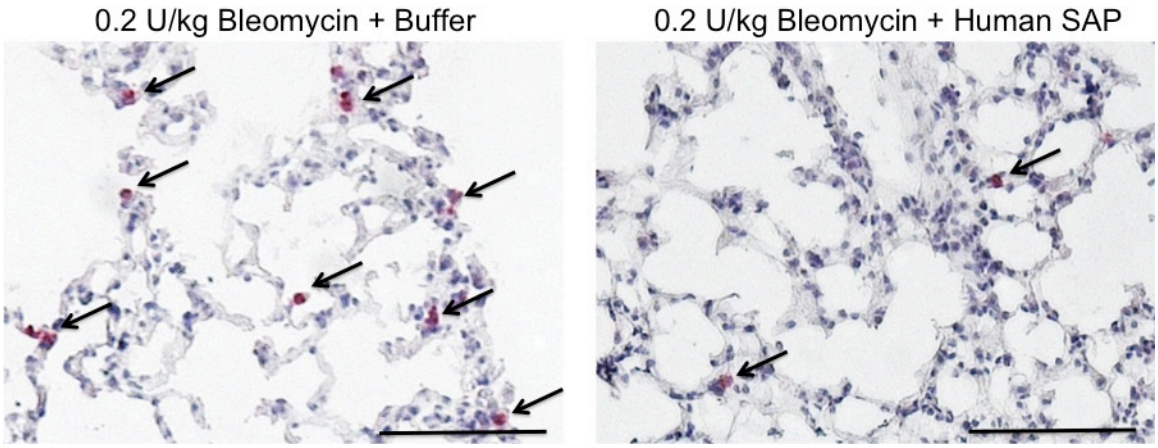
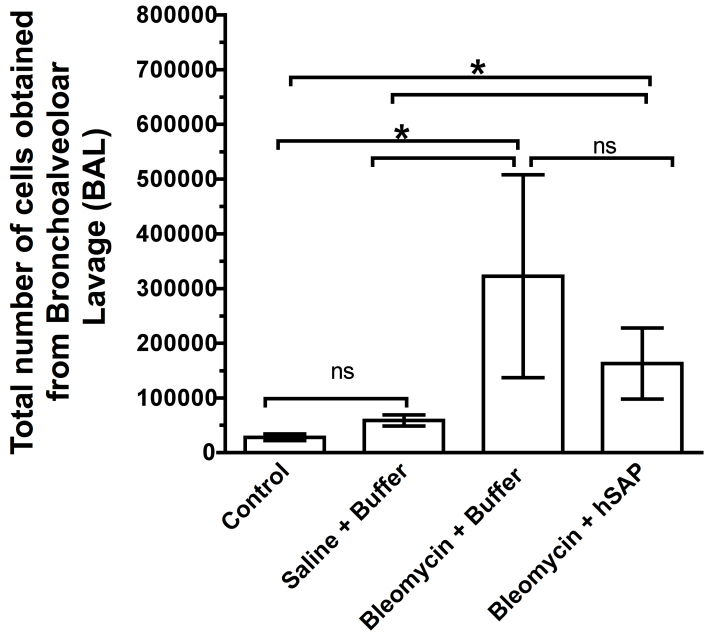


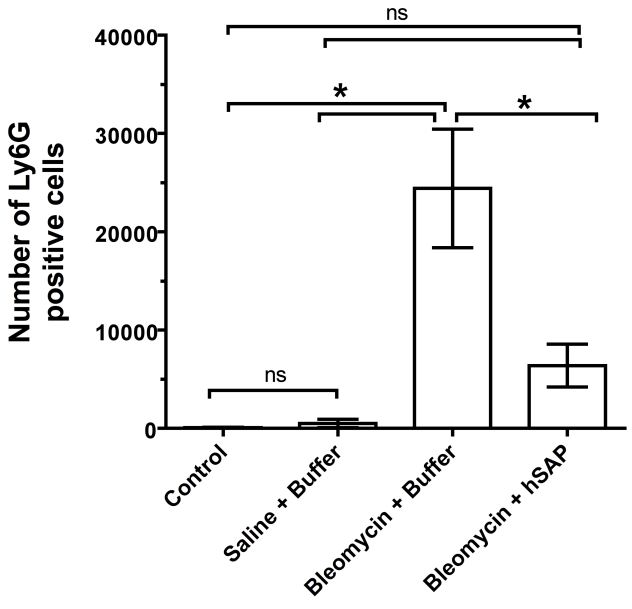
Figure 4.3.10: SAP decreases the accumulation of Ly6G-positive cells in 0.2 U/kg bleomycin-treated mouse lungs. Mice were treated with 0.2 U/kg bleomycin using an oropharyngeal technique on day 0. Mice were then injected with either 50 µg SAP or an equal volume of buffer on days 1 and 2. After the mice were sacrificed on day 3, cells were collected by BAL. (A) Total number of cells collected from BAL of untreated mice (control), mice treated with saline and buffer, mice treated with 0.2 U/kg bleomycin and buffer, mice treated with 0.2 U/kg bleomycin and 50 µg hSAP, or mice treated with bleomycin and 50 µg mSAP. (B) Total number of Ly6G-positive cells in the above experimental groups. The results are mean ± SEM of the number of cells that positively stained for Ly6G (n = 4 for control and mice treated with bleomycin and buffer, n = 5 for mice treated with saline and buffer or mice treated with bleomycin and mouse SAP, and n = 6 for mice treated with bleomycin and human SAP). Using the non-parametric Mann Whitney two tailed t-test, there was an increased number of

Ly6G-positive cells in the BAL from bleomycin and buffer when compared to control, mice treated with saline and buffer, or mice treated with bleomycin and human SAP, with * indicating $p < 0.05$. Using the non-parametric Mann Whitney two tailed t-test, there was no significant difference between mice treated with bleomycin and buffer or mice treated with bleomycin and mouse SAP. However, using the non-parametric Mann Whitney one-tailed t-test, there was a statistically significant increase in the number of Ly6G-positive cells in the BAL from mice treated with bleomycin and buffer when compared to the number of Ly6G-positive cells in the BAL from mice treated with bleomycin and mouse SAP. (C) After obtaining cells from BAL, day 3 lung sections from mice treated with 0.2 U/kg bleomycin and buffer or 0.2 U/kg bleomycin and human SAP were stained with anti-mouse Ly6G to detect neutrophils. Arrows indicate Ly6G-positive cells. Bars are 100 μm.

A


Total number of cells obtained from Bronchoalveoloar Lavage (BAL)
800000
700000
600000
500000
400000
300000
200000
100000
0
*
*
ns
ns
Control
Saline + Buffer
Bleomycin + Buffer
Bleomycin + hSAP

B


Number of Ly6G positive cells
40000
30000
20000
10000
0
ns
*
*
ns
Control
Saline + Buffer
Bleomycin + Buffer
Bleomycin + hSAP

C

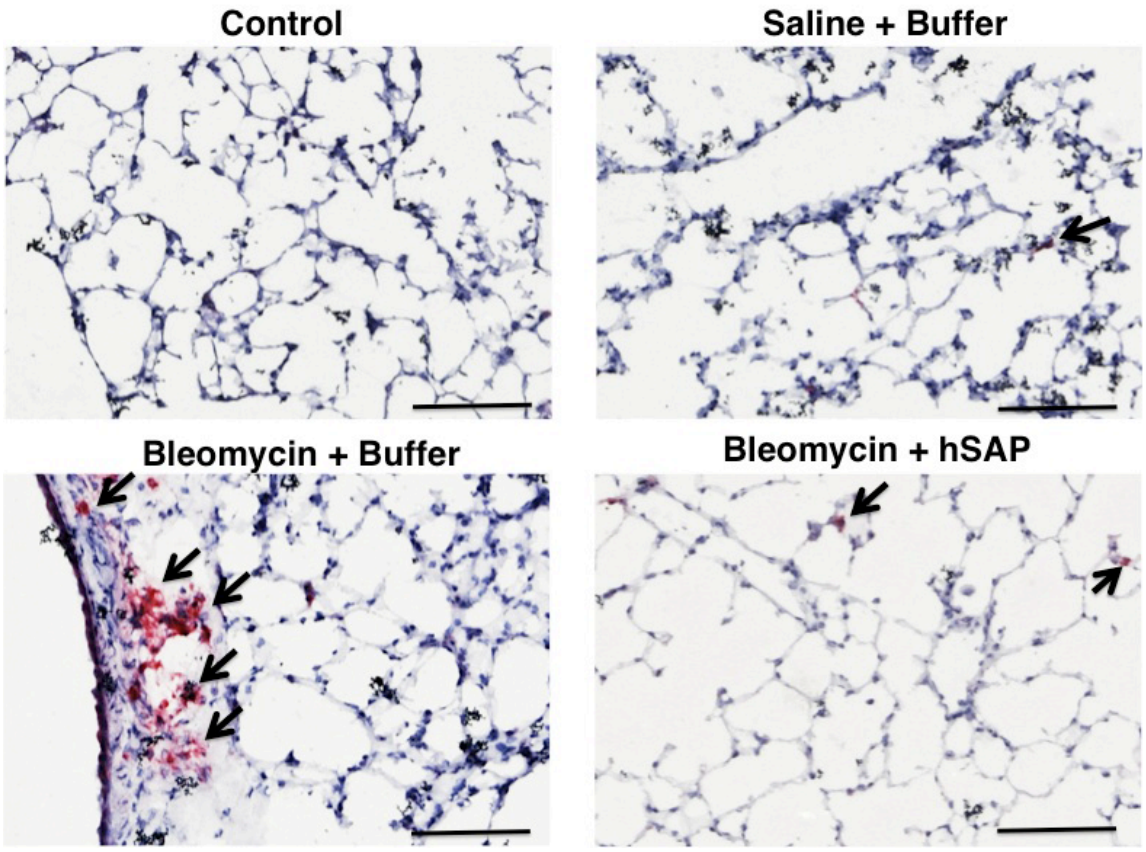


Figure 4.3.11: SAP decreases the accumulation of Ly6G-positive cells in 3 U/kg bleomycin-treated mouse lungs. Mice were treated with 3 U/kg bleomycin using an oropharyngeal technique on day 0. Mice were then injected with either 50 µg SAP or an equal volume of buffer on days 1 and 2. After the mice were sacrificed on day 3, cells were collected by BAL. (A) Total number of cells collected from BAL of untreated mice (control), mice treated with saline and buffer, mice treated with 3 U/kg bleomycin and buffer, or mice treated with 3 U/kg bleomycin and 50 µg hSAP. (B) Total number of Ly6G-positive cells in the above experimental groups. The results are mean ± SEM of the number of cells that

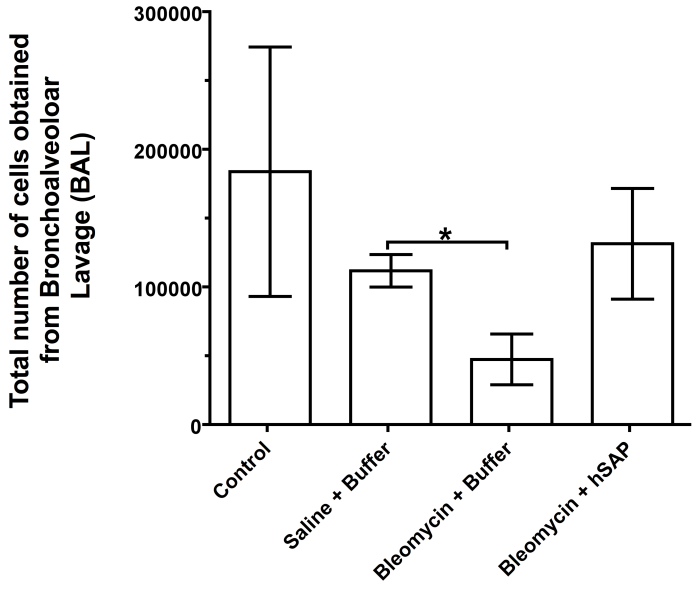
positively stained for Ly6G (n = 4 for control, mice treated with bleomycin and buffer, or mice treated with bleomycin and human SAP, and n = 5 for mice treated with saline and buffer). * indicates a significant difference with $p < 0.05$ as determined by non-parametric Mann Whitney two-tailed t-tests. (C) After obtaining cells from BAL, day 3 lung sections of untreated mice (control), mice treated with saline and buffer, mice treated with 3 U/kg bleomycin and buffer, or mice treated with 3 U/kg bleomycin and 50 µg hSAP were stained with anti-mouse Ly6G to detect neutrophils. Arrows indicate Ly6G-positive cells. Bars are 100 µm.

4.3.9 SAP inhibits the accumulation of Ly6G-positive cells in the lungs of sap$^{-/-}$ mice treated with bleomycin

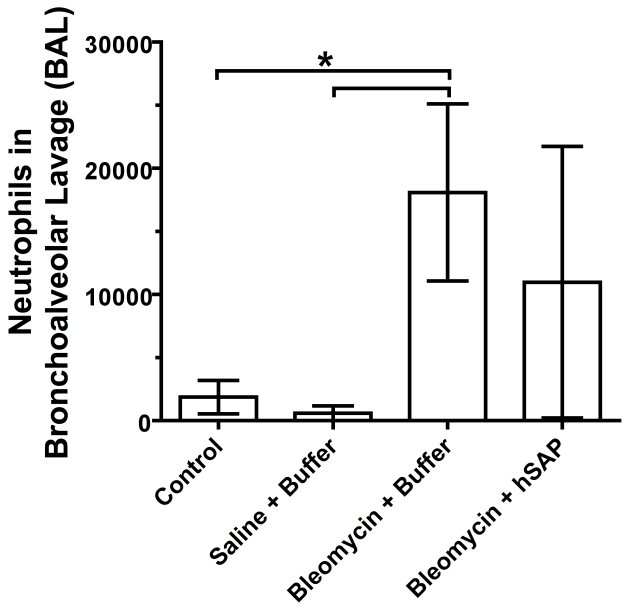
Since SAP decreases the number of neutrophils in the lungs of bleomycin-treated mice, we examined the effect of SAP on neutrophil accumulation using *sap*$^{-/-}$ mice. We treated *sap*$^{-/-}$ mice with 3 U/kg bleomycin, followed by intraperitoneal injections of 50 µl of buffer containing 50 µg human SAP, or 50 µl of buffer, after 24 and 48 hours. There was no statistically significant difference between the total number of cells collected from the BAL of control or saline-treated mice (Figure 4.3.12A). Although the number of cells obtained from the BAL of control was greater than the number of cells obtained from the BAL of mice treated with 3 U/kg bleomycin and buffer (Figure 4.3.12A), the difference was not statistically significant. However, there was a significant decrease in the number of total cells collected in the BAL from *sap*$^{-/-}$ mice treated with 3 U/kg bleomycin and buffer when compared to the total cells collected in the BAL from saline-treated mice (Figure 4.3.12A). There was no statistically significant difference between the total number of cells obtained from untreated control mice, saline-treated, or 3 U/kg bleomycin and hSAP treated mice. There was also an increased number of Ly6G-positive cells in the BAL from mice treated with 3 U/kg bleomycin and buffer when compared to the number of Ly6G-positive cells in the BAL from control or saline-treated mice (Figure 4.3.12B). However, there was no statistically significant difference in Ly6G-positive cells from the

BAL of mice treated with 3 U/kg bleomycin and human SAP when compared to the Ly6G-positive cells in the BAL from control or saline-treated mice (Figure 4.3.12B). Although there was a decreased number of Ly6G-positive cells in the BAL from mice treated with 3 U/kg bleomycin and human SAP when compared to the number of Ly6G-positive cells in the BAL from mice treated with 3 U/kg bleomycin and buffer, the difference was not statistically significant. To further examine the neutrophils accumulated in the lungs of *sap*$^{-/-}$ mice, we stained the lung sections after doing the BAL for Ly6G. We did not detect many neutrophils in the lung sections of untreated *sap*$^{-/-}$ mice or saline-treated *sap*$^{-/-}$ mice (Figure 4.3.12C). There were more neutrophils observed in the lung sections of *sap*$^{-/-}$ mice treated with bleomycin and buffer when compared to the lung sections from other treatment groups (Figures 4.3.12C). Intraperitoneal injection of human SAP decreases the number of neutrophils that accumulate in the lungs of bleomycin-treated mice (Figure 4.3.12C). Our results thus suggest that SAP reduces the bleomycin-induced accumulation of neutrophils in the lungs of mice.

A


Total number of cells obtained from Bronchoalveoloar Lavage (BAL)
300000
200000
100000
0
*
Control
Saline + Buffer
Bleomycin + Buffer
Bleomycin + hSAP

B


Neutrophils in Bronchoalveolar Lavage (BAL)
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20000
10000
0
*
Control
Saline + Buffer
Bleomycin + Buffer
Bleomycin + hSAP

C

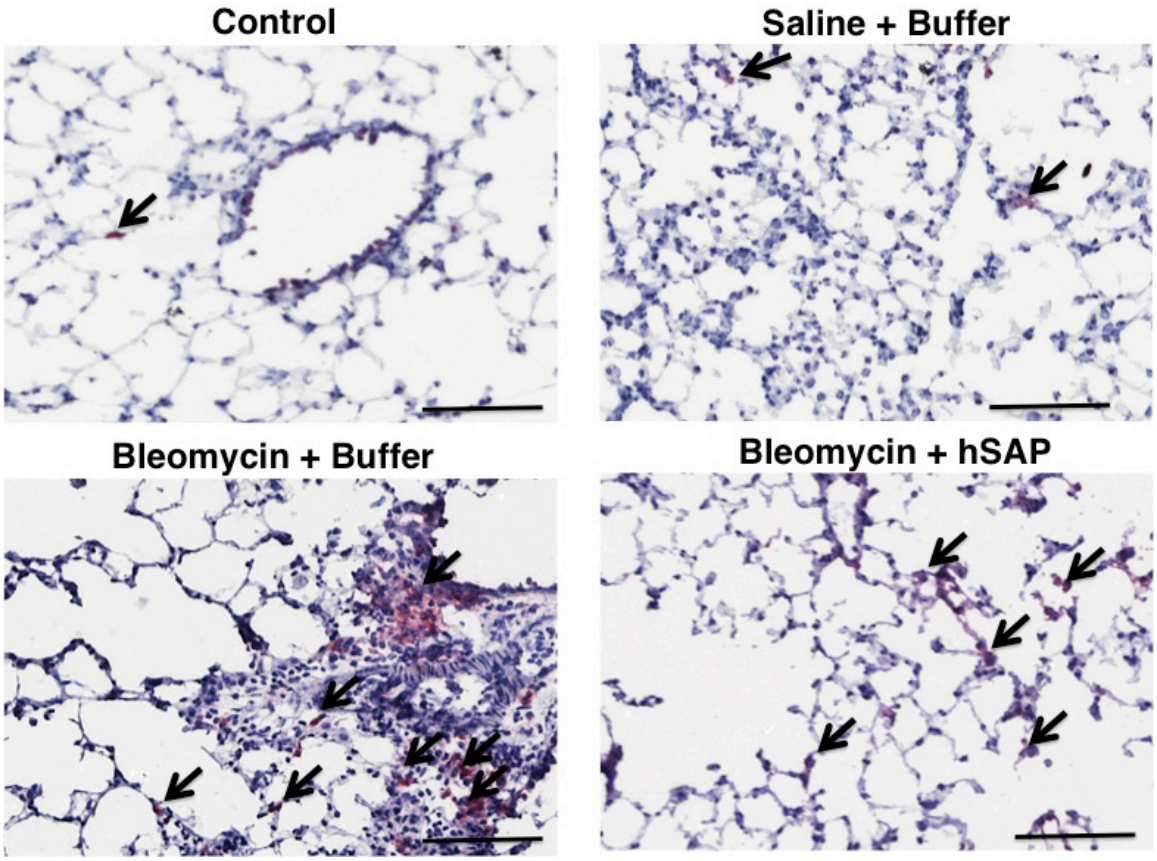

Figure 4.3.12: SAP decreases the accumulation of Ly6G-positive cells in the lungs of 3 U/kg bleomycin-treated sap$^{-/-}$ mice. *sap$^{-/-}$* mice were treated with 3 U/kg bleomycin using an oropharyngeal technique on day 0. Mice were then injected with either 50 µg SAP or an equal volume of buffer on days 1 and 2. After the mice were sacrificed on day 3, cells were collected by BAL. (A) Total number of cells collected from BAL of untreated mice (control), mice treated with saline and buffer, mice treated with 3 U/kg bleomycin and buffer, or mice treated with 3 U/kg bleomycin and 50 µg hSAP. * indicates a significant difference with $p < 0.05$ as determined by two-tailed t-tests. (B) Total number of Ly6G-positive

cells in the above experimental groups. The results are mean ± SEM of the number of cells that positively stained for Ly6G (n = 3). * indicates a significant difference with $p < 0.05$ as determined by one-tailed t-tests. (C) After obtaining cells from BAL, day 3 lung sections of untreated *sap*$^{-/-}$ mice (control), *sap*$^{-/-}$ mice treated with saline and buffer, *sap*$^{-/-}$ mice treated with 3 U/kg bleomycin and buffer, or *sap*$^{-/-}$ mice treated with 3 U/kg bleomycin and 50 µg hSAP were stained with anti-mouse Ly6G to detect neutrophils. Arrows indicate Ly6G-positive cells. Bars are 100 µm.

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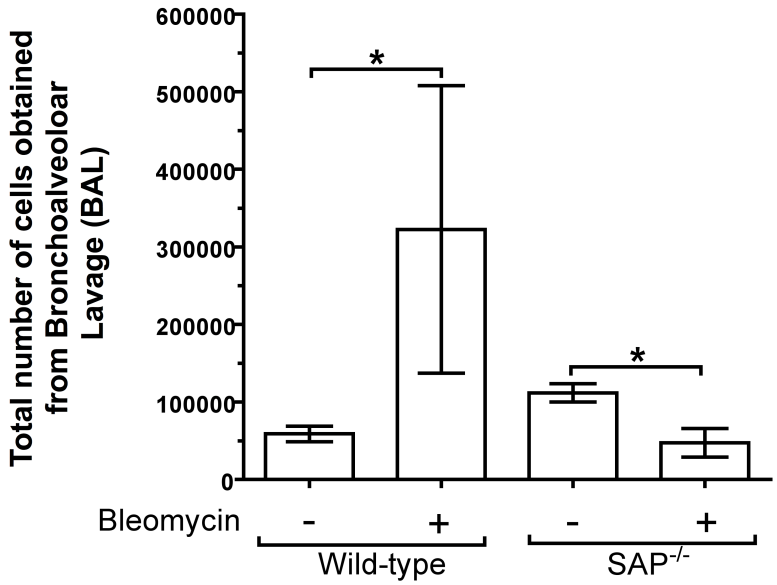
4.3.10 The lack of SAP increases the number of neutrophils that accumulates in the lungs of bleomycin-treated sap$^{-/-}$ mice compared to the bleomycin-treated wild-type mice.

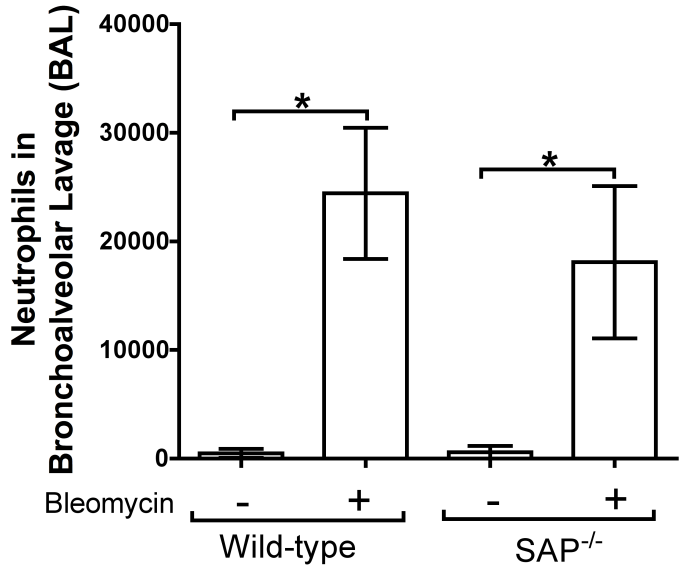
In wild-type mice, the total number of cells collected from the BAL of bleomycin-treated mice was significantly more than the total number of cells obtained from the BAL of saline-treated mice (Figure 4.3.13A). On the other hand, in *sap*$^{-/-}$ mice, the total number of cells collected from the BAL of bleomycin-treated mice was significantly fewer than the total number of cells obtained from the BAL of saline-treated mice (Figure 4.3.13B). When we stained the cells collected from the BAL for the neutrophil marker Ly6G, there was an increased number of Ly6G-positive cells in the BAL from wild-type and *sap*$^{-/-}$ mice treated with bleomycin when compared to the number of Ly6G-positive cells in the BAL from saline-treated mice (Figure 4.3.13B). However, there was no statistically significant difference in the number of neutrophils in the BALs from wild-type and *sap*$^{-/-}$ mice treated with bleomycin. Both wild-type and *sap*$^{-/-}$ mice treated with bleomycin had neutrophils remaining in the lungs after the BAL procedure (Figures 4.3.13C). However, *sap*$^{-/-}$ mice treated with bleomycin had more neutrophils remaining in the lungs compared to the wild-type mice treated with bleomycin (Figure 4.3.13C). Wild-type mice treated with bleomycin had neutrophils around the fibrotic-like lung areas, while *sap*$^{-/-}$ mice treated with bleomycin had neutrophils around the fibrotic-like lung areas and scattered around the alveolar space (Figure 4.3.13C). These data suggest that the lack of

SAP causes increased retention of neutrophils in the lungs of mice treated with bleomycin, and the decreased number of total cells in the BAL of *sap*$^{-/-}$ mice indicates that the lack of SAP causes the adherence of neutrophils in the lungs.

A


Total number of cells obtained from Bronchoalveoloar Lavage (BAL)
600000
500000
400000
300000
200000
100000
0
*
*
Bleomycin
-
+
-
+
Wild-type
SAP-/-

B


Neutrophils in Bronchoalveolar Lavage (BAL)
40000
30000
20000
10000
0
*
*
Bleomycin
-
+
-
+
Wild-type
SAP-/-

C

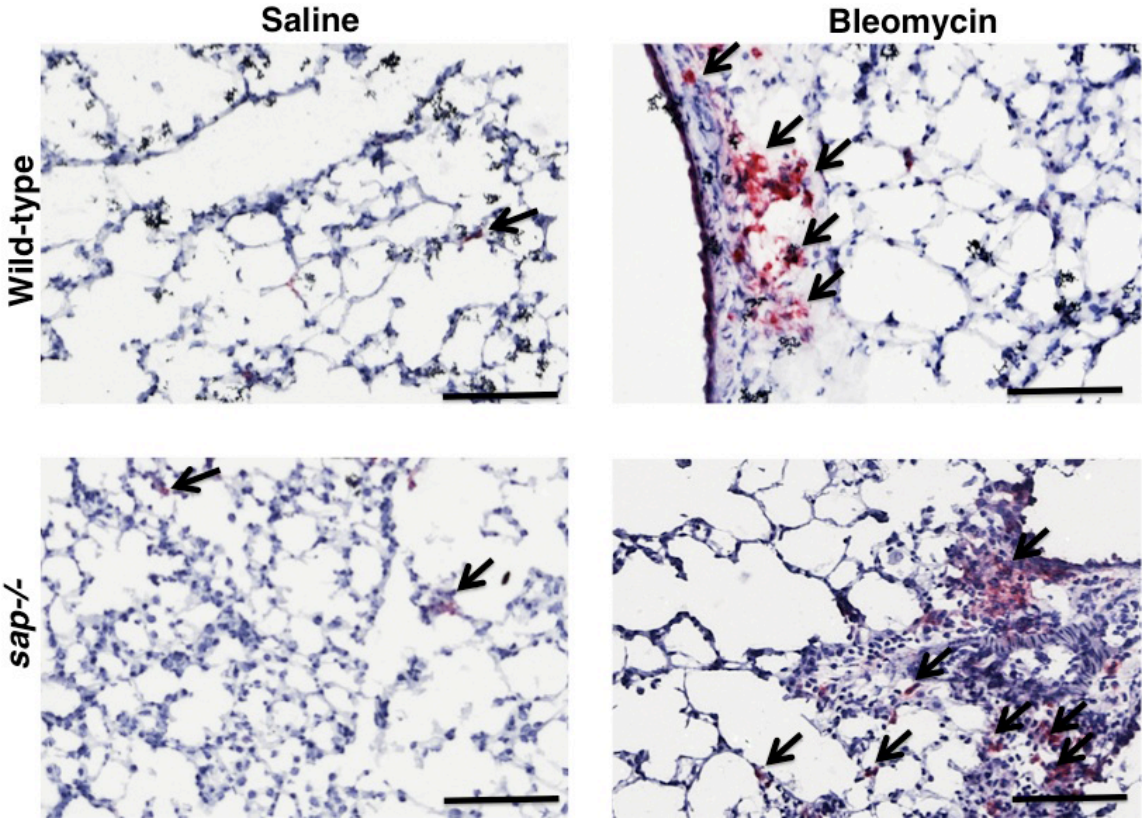


Figure 4.3.13 The lack of SAP increases the number of neutrophils remaining in the lungs of bleomycin-treated sap$^{-/-}$ mice after bronchoalveolar lavage compared to the bleomycin-treated wild-type mice. (A) Bleomycin increases the total number of cells in the BAL of wild-type mice while decreases the total number of cells in the BAL of *sap*$^{-/-}$ mice. Wild-type (C57BL6) and *sap*$^{-/-}$ mice were treated with 3 U/kg bleomycin or an equal volume of saline using an oropharyngeal technique on day 0. The mice were sacrificed on day 3, and the cells were collected by BAL. The results are mean ± SEM of the total number of cells obtained from BAL (n = 5 for wild-type mice treated with saline, n = 4 for wild-type mice treated with bleomycin, and n = 3 for *sap*$^{-/-}$ mice treated with saline or

bleomycin. * indicates a significant difference with $p < 0.05$ as determined by non-parametric Mann Whitney two-tailed t-tests for wild-type mice and one-tailed t-test for *sap*$^{-/-}$ mice. (B) Bleomycin increases the accumulation of neutrophils in the lungs of both wild-type and *sap*$^{-/-}$ mice. Mice were treated with 3 U/kg bleomycin or equal volume of saline using an oropharyngeal technique on day 0. The mice were sacrificed on day 3, and the cells were collected by BAL. The results are mean ± SEM of the total number of neutrophils obtained from BAL (n = 5 for wild-type mice treated with saline, n = 4 for wild-type mice treated with bleomycin, and n = 3 for *sap*$^{-/-}$ mice treated with saline or bleomycin. * indicates a significant difference with $p < 0.05$ as determined by non-parametric Mann Whitney two-tailed t-tests for wild-type mice and a one-tailed t-test for *sap*$^{-/-}$ mice. (C) Bleomycin increases the accumulation of neutrophils in the lungs of both wild-type and *sap*$^{-/-}$ mice. After obtaining cells from BAL, lung sections from wild-type and *sap*$^{-/-}$ mice treated with bleomycin or saline were stained with anti-mouse Ly6G to detect neutrophils. Arrows indicate Ly6G-positive cells. Bars are 100 μm.

4.4 Discussion

We found that SAP inhibits cell debris-induced neutrophil spreading and TNFα-induced neutrophil adhesion on different extracellular matrices. However, SAP has no effect on the surface levels or the intensity of neutrophil adhesion molecules such as CD11b, CD62L, CD18, or CD44 that are affected by neutrophil activating factors such as TNF-α, GM-CSF, or fMLP. SAP also has no effect on the production of hydrogen peroxide induced by neutrophil activating factors such as PMA, PDBu, fMLP, or TNF-α. In addition, SAP was unable to have any effect on fMLP induced neutrophil migration. Nevertheless, in our *in vivo* experiment, I saw increased neutrophil accumulation in the lungs of bleomycin treated mice. Intraperitoneal injection of 50 μg of SAP on day 1 and 2 significantly reduced the number of neutrophils that accumulate in the lungs of mice treated with bleomycin. I further repeated these experiments with *sap*$^{-/-}$ mice. Although not statistically significant, *sap*$^{-/-}$ mice treated with bleomycin and SAP had fewer neutrophils in the BAL than *sap*$^{-/-}$ mice treated with bleomycin. There was a significant decline in the number of neutrophils remaining in the lungs after BAL in *sap*$^{-/-}$ mice treated with bleomycin and SAP when compared to the lung sections of the *sap*$^{-/-}$ mice treated with bleomycin. There was also an increased number of neutrophils remaining in the lungs after BAL in *sap*$^{-/-}$ mice that were treated with bleomycin when compared to the wild-type mice.

Since neutrophil accumulation is a key cause of additional tissue damage in acute lung injury, it is important to find therapies that would prevent this accumulation. We have now found that SAP can be used as a therapy in an animal model to prevent neutrophil accumulation. Our hypothesis is that SAP acts as an anti-adhesive agent, and therefore, inhibits the accumulation of neutrophils in the injured lungs. There are other pentraxin family proteins that also inhibit neutrophil accumulation in animal models of ALI/ARDS (Heuertz and Webster, 1997). The pentraxin proteins such as C-reactive protein (CRP) and the pentraxin PTX3 also prevent the number of neutrophils that accumulate in injured lungs (Deban et al., 2010; Heuertz and Webster, 1997). CRP inhibits neutrophil adhesion and chemotaxis, and limits the number of neutrophils that accumulate in injured lungs (Heuertz and Webster, 1997). Administering CRP intravenously 10 minutes before the intratracheal instillation of the neutrophil chemotactic agent C5a dramatically reduced the neutrophil accumulation in lungs when examined four hours later (Heuertz et al., 1993). CRP inhibits L-selectin-mediated neutrophil adhesion on TNF-α activated endothelial cells by inducing L-selectin shedding from neutrophils. Further, CRP peptide 201-206 is the critical peptide that mediates anti-adhesive action through CD32 (El Kebir et al., 2011). Both native and CRP peptide 201-206 prevent neutrophil chemotaxis towards fMLP by inhibiting fMLP-induced p38 MAP kinase activity (Heuertz et al., 1999). Similarly, the pentraxin PTX3 also prevents neutrophil recruitment *in vivo* (Deban et al., 2010). Pre-treating mice intravenously with PTX3 reduced the number of

neutrophils in acid-induced acute lung injury in mice when examined 3 hours later (Mantovani 2010). PTX3 deficiency also increases the number of neutrophils in the lungs of mice treated with lipopolysaccharide (LPS) (Han et al., 2011). PTX3 blocks the interaction of P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils from interacting with P-selectin on the activated endothelial cells and causes neutrophil detachment rather than arrest to prevent neutrophil migration (Deban et al., 2010).

We have now found that SAP also prevents the accumulation of neutrophils in the lungs of bleomycin-injured mice. Since SAP inhibits the adhesion of TNF-α-induced neutrophil adhesion, it is probable that SAP reduces the accumulation of neutrophils by dampening the interaction of neutrophils with extracellular matrices. We still do not know the neutrophil adhesion receptors that are affected by SAP. We have examined integrin molecules such as CD11b and CD18, however, we have not examined beta1 integrin molecules such as $\alpha2\beta1$, $\alpha4\beta1$, $\alpha5\beta1$, $\alpha6\beta1$, and $\alpha9\beta1$ that are found in neutrophils and can recognize different extracellular matrices (Birner et al., 1999; Gao and Issekutz, 1997; Lundberg et al., 2006; Mambole et al., 2010). Further work is needed to understand SAP-induced signaling that prevents TNF-α-induced neutrophil adhesion and prevents neutrophil accumulation in the bleomycin-injured mice lungs. Nevertheless, the current findings still show the possible therapeutic effect

of SAP on limiting neutrophil accumulation in acute respiratory distress syndrome.

Concluding Remarks

Fibrosing diseases and ARDS are major debilitating diseases with high mortality rates in many developed countries (King et al., 2011; Perez et al., 2003; Wheeler and Bernard, 2007). There are no effective therapies for fibrosing diseases or ARDS, therefore, finding therapies for fibrosing diseases and ARDS would help many patients and families. Inhibiting monocyte-derived cells called fibrocytes is one of the ways to reduce the progression of fibrosing diseases. The accumulation of immune cells called neutrophils is a major factor that worsens the lung injury in ARDS patients, therefore, inhibiting the accumulation of neutrophils in the lungs of ARDS patients is one of the ways to reduce the progression of ARDS. In this thesis, I have examined the regulation of fibrocyte differentiation and found that a plasma protein called serum amyloid P that already has a potential therapy for fibrosis can also be used as a therapy for ARDS.

In our laboratory, we try to find fibrosing disease therapies by examining factors that regulate fibrocyte differentiation. Since Toll-like receptors (TLRs) are present on monocytes, and pathogens that can infect a wound have and/or release TLR agonists, in chapter 2, I examined whether TLR agonists affect fibrocyte differentiation. When human peripheral blood mononuclear cells (PBMCs) were cultured with TLR3, TLR4, TLR5, TLR7, TLR8 or TLR9 agonists,

there was no significant effect on fibrocyte differentiation, even though enhanced extracellular tumor necrosis factor (TNF)-α accumulation and/or increased cell surface CD86 or antigen presenting cell marker major histocompatibility complex (MHC) class II levels were observed. However, all TLR2 agonists tested inhibited fibrocyte differentiation without any significant effect on cell survival. Adding TLR2 agonists to purified monocytes had no effect on fibrocyte differentiation. However, some TLR2 agonists caused PBMCs to secrete a factor that inhibits the differentiation of purified monocytes into fibrocytes. This factor is not interferon (IFN)-α, IFN-γ, interleukin (IL)-12, aggregated immunoglobulin G (IgG) or serum amyloid P (SAP), factors known to inhibit fibrocyte differentiation. TLR2 agonist-treated PBMCs secrete low levels of IL-6, TNF-α, IFN-γ, granulocyte colony-stimulating factor and transforming growth factor (TGF)-β1, but combinations of these factors had no effect on fibrocyte differentiation from purified monocytes. Our results indicate that TLR2 agonists indirectly inhibit fibrocyte differentiation and that, for some TLR2 agonists, this inhibition involves other cell types in the PBMC population secreting an unknown factor that inhibits fibrocyte differentiation. Together, these data suggest that the presence of some bacterial signals can inhibit fibrocyte differentiation and may thus slow wound closure. The biggest challenge in this project was to find the secreted factor that inhibits fibrocyte differentiation. It was difficult to purify the secreted factor because I could not generate large quantities of conditioned media from PBMC incubated with TLR2 agonists since I was working with human PBMC.

In chapter 3, I have shown that HMWHA potentiates the differentiation of human monocytes into fibrocytes, while LMWHA inhibits fibrocyte differentiation. Digestion of HMWHA with hyaluronidase produces small hyaluronic acid fragments, and these fragments inhibit fibrocyte differentiation. Monocytes internalize HMWHA and LMWHA equally well, suggesting that the opposing effects on fibrocyte differentiation are not due to differential internalization of HMWHA or LMWHA. Adding HMWHA to PBMC does not appear to affect the levels of the hyaluronic acid receptor CD44, whereas adding LMWHA decreases CD44 levels. The addition of anti-CD44 antibodies potentiates fibrocyte differentiation, suggesting that CD44 mediates at least some of the effect of hyaluronic acid on fibrocyte differentiation. The fibrocyte differentiation-inhibiting factor serum amyloid P (SAP) inhibits HMWHA-induced fibrocyte differentiation and potentiates LMWHA-induced inhibition. Conversely, LMWHA inhibits the ability of HMWHA, interleukin-4 (IL-4), or interleukin-13 (IL-13) to promote fibrocyte differentiation. We hypothesize that hyaluronic acid signals at least in part through CD44 to regulate fibrocyte differentiation, with a dominance hierarchy of SAP > LMWHA ≥ HMWHA > IL-4 or IL-13. The biggest challenge in this project was to figure out why HMWHA potentiates fibrocyte differentiation while LMWHA inhibits fibrocyte differentiation even though they are both composed of the same chemical structure. Many researchers have seen opposite effects of HMWHA and LMWHA on different types of cells (Bollyky et

al., 2007; McKee et al., 1996), but there are no known mechanisms to explain why a simple polysaccharide could have opposite effects.

In chapter 4, examining the effect of SAP on neutrophils led us to the exciting possibility of using SAP as a therapy for ARDS. We found that SAP inhibits cell debris-induced neutrophil spreading and TNFα-induced neutrophil adhesion on different extracellular matrices. Further, *in vivo*, we found that intraperitoneal injection of exogenous SAP inhibits the accumulation of neutrophils induced by bleomycin in both wild-type and *sap*$^{-/-}$ mice. However, SAP has no effect on the classical neutrophil adhesion molecules, production of hydrogen peroxide, or the migration of neutrophils. The biggest challenge in this project was to find the adhesion molecules that were affected by SAP. SAP inhibits TNF-α-induced neutrophil adhesion on plate assays, but it was difficult to mimic this interaction in solution and examine neutrophil adhesion molecules. In the future, we could examine other neutrophil adhesion molecules that may be affected by SAP. We could also examine the effect of SAP on TNF-α-induced neutrophil adhesion in a static condition rather than a non-static condition to truly observe the detachment of neutrophils caused by SAP. Since C-reactive protein (CRP) affects a MAP kinase pathway, we could also examine the effect of SAP on this pathway.

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